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(71) Applicant *(for all designated States except US)*: REGENTS OF THE UNIVERSITY OF MINNESOTA [US/US]; 100 Church Street, S.E., Minneapolis, MN 55455 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: HSIAO, Karen [US/US]; 7 Oriole Lane, North Oaks, Minneapolis, MN 55127 (US). BORCHELT, David, R. [US/US]; 723 Stevenson Lane, Baltimore, MD 21286 (US). SISODIA, Sangra, M. [US/US]; 416 Old Trail, Baltimore, MD 21212 (US).

(74) Agents: RAE-VENTER, Barbara et al.; Weil, Gotshal & Manges, Suite 280, 2882 Sand Hill Road, Menlo Park, CA 94025 (US).

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(54) Title: TRANSGENIC NON-HUMAN MAMMALS WITH PROGRESSIVE NEUROLOGIC DISEASE

(57) Abstract

Provided is a transgenic non-human eukaryotic animal whose germ cells and somatic cells contain the amyloid precursor protein sequence introduced into the animal, or an ancestor of the animal, at an embryonic stage. In mice, an age-related CNS disorder characterized by agitation, neophobia, seizures, inactivity, diminished cerebral glucose utilization, cortico-limbic gliosis, and death, develops. An acceleration of this disorder occurs in transgenic mice expressing human and mouse Alzheimer amyloid precursor proteins (APP) produced using a hamster prion protein gene-derived cosmid vector that confers position-independent, copy number-dependent expression. In transgenic mice the disorder develops in direct relationship to brain levels of transgenic APP, but mutant APP confers the phenotype at lower levels of expression than wild-type APP. The disorder occurs in the absence of extracellular amyloid deposition, indicating that some pathogenic activities of APP are dissociated from amyloid formation.

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TRANSGENIC NON-HUMAN MAMMALS WITH PROGRESSIVE NEUROLOGIC DISEASE

Introduction

Technical Field:

The invention relates to transgenic animals with a progressive degenerative neurologic disease.

Background:

The term degenerative as applied to diseases of the nervous system is used to designate a group of disorders in which there is gradual, generally relentlessly progressing wasting away of structural elements of the nervous system; many of the conditions so designated depend upon abnormal genetic factors. The degenerative diseases manifest themselves by a number of syndromes distinguished by their clinical and pathological features. Nevertheless, there are certain aspects common to all. These aspects include having a gradually progressive course of onset, bilaterally symmetric distribution of the changes brought about by the disease, and in many cases, the almost selective involvement of anatomically or physiologically related systems of neurons. Typically the pathologic process is one of slow invaluation of nerve cell bodies or their prolongations as nerve-fibers.

Amongst the degenerative diseases of the nervous system are syndromes in which the outstanding feature is progressive dementia; the syndromes in this group include senile dementia and Alzheimer's disease. Senile dementia is a fairly frequent condition of old age. Alzheimer's disease is a pathologically identical, but much more infrequent, progressive dementia which comes on well before the senile period. The distinction between the two conditions is purely clinical; pathologically they differ only in that the characteristic abnormalities tend to be more sever and widespread in cases of Alzheimer's disease and to begin at an earlier age than at the senile period.

Alzheimer's disease (AD) shows a slowly progressive mental deterioration with failure of memory, disorientation and confusion leading to profound dementia. The disease predominantly involves limbic and cortical regions of the

brain. There are several histologic features, but two are striking. First, argyrophilic plaques comprised of the amyloidogenic Aß fragment of amyloid precursor protein (APP) are scattered throughout the cerebral cortex and hippocampus. Second, neurofibrillary tangles are found in pyramidal neurons predominantly located in the neocortex, hippocampus, and nucleus basalis of Meynert. There are other changes, also. Granulovacuolar degeneration in the pyramidal cells of the hippocampus has been considered by some to be more specific for AD than plaques or neurofibrillary tangles. Finally, there is neuronal loss and gliosis in the cortex and hippocampus.

There are patients with dementia who lack the pathologic features of AD (and by definition have a different disease), and conversely, there are individuals with many of the pathologic features of AD who were not demented prior to death. The diagnosis of AD requires that both the clinical and the pathological features characteristic for the disease be present in the patient; the diagnosis cannot be made with certainty from either clinical or pathological features alone. Whether neural dysfunction and clinical abnormalities precede the development of these pathologic features, particularly the amyloid plaques and neurofibrillary tangles, is unknown.

The clinical manifestations of AD predict the regions of affected brain structures in the forebrain, including the cerebral cortex, hippocampus, amygdala, and parahippocampal gyri. These regions are known as the cortico-limbic areas of the brain. The hindbrain is spared, including the cerebellum, the pontine and the medullary nuclei. Within the cerebral neocortex, the primary cortical area is relatively spared, which corresponds to the relative clinical sparing of basic motor and sensory cortical functions.

Research into progressive neurologic disorders such as AD has been seriously impeded by the lack of easily accessible animal models. Some aspects of the neuropathology of aged primates are similar to human AD (Price, et al., (1992) *J. Neurobiol.* 23:1277-1294). Aged primates develop amyloid plaques and forme fruste neurofibrillary tangles. No other animals studied develop disease resembling AD as closely as aged primates. However, aged primates are impractical to study in large numbers; their use raises both moral and economic issues.

Transgenic mice harboring APP transgenes have been described; however, the reported transgene product expression falls considerably short of endogenous levels of APP (total APP levels in the other transgenic mice have not exceeded 150% of endogenous levels), and fails to generate a disease phenotype with a progressive neurobehavioral disorder accompanied by pathology in the cortico-limbic regions of the brain. In these other transgenic mice, there have been no signs of a progressive neurologic disorder or of neuropathologic changes in the brain which may be regarded as evidence of a true neurologic disease. Missense point mutations in the gene coding for the amyloid precursor proteins have been linked to familial AD. However, despite the discovery of disease associated mutations in APP, most published attempts to create transgenic animals with AD have involved only wild-type APP transgenes in mice (Kawabata et al., (1991) *Nature* 354, 476-478; Quon et al., (1991); *Nature* 352, 239-41.; Wirak et al., (1991) *Science* 253, 323-325; Kammesheidt et al., (1992) *Proc Natl Acad Sci U.S.A.* 89, 10857-61; Lamb et al., (1993) *Nature Genetics* 5, 22-30.) Unfortunately, several of the published studies purporting pathology have been confounded by inadequate documentation of transgene product expression and/or misinterpretation of pathology. Two have been retracted (Kawabata, et al., 1991; Wirak, et al., 1991).

Previous efforts to create a model of AD in transgenic mice have been discouraging. In most cases, transgene product expression comparable to or exceeding endogenous levels of APP was not achieved and the transgenes did not encode mutated APP. PCT/US92/11276 reports methods for using mutant genes. In some cases, the entire APP gene was not expressed, just the carboxyl terminus (Kammesheidt, et al., (1992) *Proc Natl Acad Sci U.S.A.* 89, 10857-61); this may overlook any biologic effects that the rest of the APP molecule may exert in AD.

Preamyloid APP plaques have been observed in some transgenic mice. However, preamyloid APP plaques are not necessarily indicative of a disease, since they are routinely observed in human brain regions, such as the cerebellum, which are devoid of other signs of pathology or clinical manifestations. Increased APP immunoreactivity located within vesicular structures in hippocampal neurons of transgenic mice has been reported, but the significance of this immunoreactivity

is unclear since the mice exhibited neither a progressive neurobehavioral disorder nor evidence of true neuropathology.

In general, the ceaselessly progressive course of the neurodegenerative disease is uninfluenced by current treatment modalities. It therefore is of interest to develop a transgenic nonhuman mammalian model for degenerative neurologic diseases such as senile dementia and AD wherein the animal develops a progressive degenerative neurologic disease of the cortico-limbic brain resembling the disease, both clinically and pathologically (e.g. the gliosis and the specific brain regions affected). It also is desirable that the animal develops neurologic disease within a fairly short period of time from birth, facilitating the analysis of multigenerational pedigrees. The model can be used to study the pathogenesis and treatment of degenerative neurologic diseases since there is a distinct and robust clinical and pathologic phenotype to examine and score.

Relevant Literature:

Quon et al. (1991) *Nature* 352:239 describe transgenic mice containing human amyloid precursor protein genes. Lamb et al. (1993) *Nature Genetics* 5:22 describe transgenic mice in which the amount of amyloid precursor protein expressed is approximately 50% over endogenous levels. PCT application US 92/11276 discloses methods for constructing transgenic mice and rats which would express, under various promoters, three forms of the B-amyloid precursor protein (APP), APP 69S' APP 751, and APP 770. No data are provided in the specification as to whether expression is obtained in vivo using these methods.

Other transgenic mouse studies of Alzheimer amyloid precursor (APP) protein include the following. (Greenberg, B.D. (1993) Abstract 421.12, *Society for Neuroscience Abstracts* 19:1035.) The APP protein gene was expressed using MAPP and mMt-I promoters. Schwartz, et al. ((1993) Abstract 421.13, *Society for Neuroscience Abstracts*,19:1035) disclose neuron-specific expression of human beta-amyloid precursor protein (APP) in transgenic mice. Savage, et al. ((1993) Abstract 421.14 *Society for Neuroscience Abstracts* 19:1035) disclose 5 human amyloid precursor protein expression in transgenic mice as a model of Alzheimer's disease: search for pathology. Lieberburg, I. ((1993) Abstract 421.15, *Society for Neuroscience Abstracts* 19:1035) disclose expression of human protein in

transgenic mice using the NSE promotor. Fukuchi, K. et al. ((1993) Abstract 421.16, *Society for Neuroscience Abstracts* 19:1035) disclose intestinal beta-amyloidosis in transgenic mice. A chicken beta-actin promotor and CMV enhancer were used for expressing the APP protein gene.

Wagner et al. ((1981) *Proc. Nat. Acad. Sci. U.S.A.* 78:5016) describe transgenic mice containing human globin genes. Scott et al. ((1989) *Cell* 59: 847) describe transgenic mice containing hamster prion protein genes. Hsiao et al. ((1990) *Science* 250:1587) describe transgenic mice containing mutant human prion protein genes. Hsiao disclosed a model for Gerstmann-Straussler-Scheinker disease (GSS), a rare neurodegenerative disease caused by mutations in the prion protein (PrP) gene, in transgenic mice in which levels of mutant transgene product exceeding endogenous levels were needed to generate a clinical and pathological phenotype (Hsiao et al., (1990) *Science* 250:1587-1590); Hsiao, et al., (1994) *Proc. Natl. Acad. Sci. USA*, 91:9126-9130).

SUMMARY OF THE INVENTION

A transgenic non-human animal model for progressive neurologic disease is provided, together with methods and compositions for preparation of the animal model and methods for using it. The non-human mammals are obtained by the steps of introducing multiple copies of an expression cassette into the non-human mammal at an embryonic stage, and developing the embryo to term in a pseudo-pregnant foster female. The expression cassette comprises an amyloid precursor protein coding sequence operably joined to regulatory sequences for expression of the coding sequence in neurologic tissues at a level at least two to four-fold that of endogenous levels of wildtype amyloid precursor protein. The resulting transgenic non-human mammals develop progressive neurologic disease in the cortico-limbic areas of the brain. The transgenic animals find use for example in screening protocols for treatment and prevention of progressive neurologic diseases.

DRAWINGS

Figure 1 is a diagrammatic representation of a HuAPP cDNA sequence.

Figure 2 is a diagrammatic representation of different APP sequences which can be expressed in transgenic animals (not exhaustive).

Figure 3 is a diagrammatic representation of a hamster PrP cosmid vector with a tetracycline-resistance sequence flanked by SalI sites replacing the PrP coding sequence.

Figures 4 and 5 are diagrammatic representations of a hamster PrP cosmid vector fused with HuAPP sequences modified for strong translation initiation as illustrated in Figures 6 and 7.

Figures 6 and 7 are diagrammatic representations of HuAPP sequences modified for strong translation initiation and flanking SalI restriction sites.

Figure 8 is a diagrammatic representation of PCR primers which can be used to detect transgenes.

Figure 9 shows age-related CNS dysfunction in transgenic and non-transgenic FVB mice. In two lines of Tg mice, Tg(HuAPP695.TRImyc)1130H and Tg(HuAPP695.TRImyc)1118 expressing variant HuAPP at 3.6 and 1.4 times endogenous MoAPP levels, respectively, the average onset of illness was inversely related to APP levels. A subset of Tg(HuAPP695.WTmyc)1874 mice and non-Tg mice developed clinical and pathological abnormalities similar to those in affected Tg mice, but with significantly lower penetrance at any given age.

Figure 10 shows cortico-limbic hypertrophic astrocytic gliosis in transgenic and non-transgenic FVB mice exhibiting behavioral abnormalities. Coronal sections of cortico-limbic and brainstem structures reacted with antibody to GFAP show hypertrophic gliosis in cortico-limbic areas of animals exhibiting behavioral abnormalities. Figure 10A, Tg(HuAPP695.TRImyc)1118-334 exhibiting behavioral abnormalities (agitation and low corner index scores) at 144 days of age, sacrificed at 206 days; Figure 10B, non-Tg littermate of Tg1118-334 without behavioral abnormalities, age 206 days; Figure 10C, non-Tg #4565 exhibiting behavioral abnormalities (inactivity and low corner index scores) at 324 days of age, sacrificed at 334 days; Figure 10D, non-Tg littermate of #4565 without behavioral abnormalities, age 334 days.

Figure 11 shows transgenic HuAPP protein expression in brain tissue. HuAPP protein expression was measured in a semi-quantitative fashion in four lines of Tg mice, Tg(HuAPP695.Wtmyc)466, Tg(HuAPP695.TRImyc)1056, Tg(HuAPP695.TRImyc)1118, Tg(HuAPP695.TRImyc)1130H, harboring 40, 7, 21 and 74 transgene copy numbers, respectively. Relative levels of transgenic

compared with endogenous brain MoAPP were examined by immunoblot analysis with two polyclonal APP antisera, CT15 (Figure 11A) and anti-GID (Figure 11A), and a monoclonal antibody, 22C11 (Figure 11B). Ct15 antiserum recognized the C-terminal 15 amino acids of APP, a region in which mouse and human APP are homologous. GID antiserum recognizes an epitope 175-186 residues from the amino terminus of APP695, a region in which mouse and human APP are identical. Equivalent amounts of protein from detergent-extracted brain homogenates of non-Tg and Tg littermates were immunoblotted in parallel. Primary antibody was revealed by ^{125}I-protein A. For monoclonal antibodies, blots were first incubated with rabbit antiserum to mouse IgG. The amount of bound ^{125}I-protein A was quantified using a phosphorimager, demonstrating a direct relationship between transgene copy number and transgene product expression. To measure the level of HuAPP specifically, brain homogenates were probed with 6E10 antibody raised against residues 1-17 of human AB (Kim, et al. (1990) *Neuroscience Research Communications* 7, 113-122). Figure 11c shows the regional expression of HuAPP in the brain. The relative amount of HuAPP in 10% w/v homogenates of various tissues was specifically detected in Tg(HuAPP695.TRImyc)1130H mice using the 6E10 antibody. Equivalent amounts of protein were immunoblotted in each lane. Lanes 1, telencephalon; 2, diencephalon; 3, mesencephalon; 4, pons; 5, cerebellum; 6, mudulla; 7, spinal cord. The highest HuAPP level, in the telencephalon, was approximately twice that of the cerebellum.

Figure 12 shows the dependence of transgenic brain APP expression upon species and copy number.

Figure 13 shows HuAPP expression in neurons of transgenic mice. Figure 13A, Tg, formic acid pretreatment, 6E10 antibody (hippocampus); Figure 13B, Non-Tg, formic acid pretreatment, 6E10 antibody (hippocampus); Figure 13C Tg, formic acid pretreatment, 6E10 antibody (cerebral cortex); Figure 13D, AD plaque, formic acid pretreatment, 6E10 antibody; Figure 13E, AD plaque, no formic acid pretreatment, 6E10 antibody; Figure 13F, AD plaque, microwave pretreatment, 8E5 antibody; Figure 13G, Tg, microwave pretreatment, 8E5 antibody (hippocampus); Figure 13H, Non-Tg, microwave pretreatment, 8E5 antibody (hippocampus).

Figure 14 shows the dependence of the CNS disorder upon level of transgenic brain APP expression and APP genotype.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The invention is directed to a transgenic nonhuman eukaryotic animal, preferably a rodent, such as a mouse, together with methods and compositions for preparing and using the animal. The animal expresses an amyloid precursor protein (APP) sequence at a level in brain tissues such that the animal develops a progressive neurologic disorder within a short period of time from birth, generally within a year from birth, preferably within 2 to 6 months, from birth. The APP protein sequence is introduced into the animal, or an ancestor of the animal, at an embryonic stage, preferably the one cell, or fertilized oocyte, stage, and generally not later than about the 8-cell stage. The zygote or embryo is then developed to term in a pseudo-pregnant foster female. The amyloid precursor protein genes are introduced into an animal embryo so as to be chromosomally incorporated in a state which results in the supra-endogenous expression of the amyloid precursor protein and the development of a progressive neurologic disease in the cortico-limbic areas of the brain, areas of the brain which are prominently affected in progressive neurologic disease states such as AD.

The present invention offers several advantages over existing models for progressive neurologic disorders such as AD. The transgenic animals express high levels of either native APP or mutant APP and develop a neurologic illness accompanied by premature death. Gliosis and intracellular APP/Aß accretions are present in the hippocampus and cerebral cortex. The gliosis and clinical manifestations in affected transgenic animals are indicative of a true neurologic disease. The progressive aspects of the neurologic disease are characterized by diminished exploratory/locomotor behavior and diminished 2-deoxyglucose uptake/utilization and hypertrophic gliosis in the cortico-limbic regions of the brain. Further, the changes that are seen are similar to those that are seen in some aging animals.

Transgenic animals of the invention are constructed using an expression cassette which includes in the 5'-3' direction of transcription, a transcriptional and translational initiation region associated with gene expression in brain tissue, DNA

encoding a mutant or wild-type an APP, and a transcriptional and translational termination region functional in the host animal. One or more introns may also be present.

For expression, of particular interest are initiation regions (also sometimes referred to as "promoters") which provide for preferential or at least substantially specific expression in brain as compared to other tissue. By "at least substantially" is intended that expression in brain tissue is greater than about 10 fold that in other tissue. Within the brain, of particular interest is expression in the cortico-limbic area. The transcriptional initiation region may be endogenous to the host animal or foreign or exogenous to the host animal. By foreign is intended that the transcriptional initiation region is not found in the wild-type animal host into which the transcriptional initiation region is introduced. By endogenous, is intended sequences both indigenous (i.e. natural to) the host animal and those present in the host animal as a result of an infectious disease, e.g. viral, prion, and the like.

The promoter preferably comprises a transcriptional initiation regulatory region and translational initiation regulatory region of untranslated 5' sequences, "ribosome binding sites", responsible for binding mRNA to ribosomes and translational initiation. The transcriptional initiation regulatory region may be composed of *cis*-acting subdomains which activate or repress transcription in response to binding of transacting factors present in varying amounts in different cells. It is preferred that all of the transcriptional and translational functional elements of the initiation control region are derived from or obtainable from the same gene. In some embodiments, the promoter is modified by the addition of sequences, such as enhancers, or deletions of non-essential and/or undesired sequences. By "obtainable" is intended a promoter having a DNA sequence sufficiently similar to that of a native promoter to provide for the desired specificity of transcription of a DNA sequence of interest. It includes natural and synthetic sequences as well as sequences which may be a combination of synthetic and natural sequences.

Tissue-specific transcription suggests that gene regulatory proteins may be bound to enhancer sequences and other upstream promoter elements. By enhancer element ("enhancer") is intended a regulatory DNA sequence that is capable of activating transcription from a promoter linked to it with synthesis beginning at the normal RNA start site; which is capable of operating in both orientations (normal or flipped); and which is capable of functioning even when moved either upstream or downstream from the promoter. Both enhancers and other upstream promoter elements bind sequence specific DNA binding proteins that mediate their effects. To identify the exact nucleotide sequences important for the function of the enhancers(s), and other upstream elements, fragments of the untranslated 5'-region encoding a protein expressed in a tissue of interest are screened for their capacity to bind nuclear proteins and for their ability to function with a heterologous promoter. Binding experiments with nuclear proteins from brain tissue can be used to determine the presence of enhancer and silencer sequences; the protein binding studies can be used to pinpoint specific nucleotide sequences that bind to a corresponding series of gene regulatory proteins.

The activity of each enhancer and other upstream promoter elements generally is present on a segment of DNA which may contain binding sites for multiple proteins. The binding sites can generally be dissected by preparing smaller mutated versions of the enhancer sequence joined to a reporter gene whose product is easily measured. The effect of each mutation on transcription can then be tested. Alternatively, fragments of this region can be prepared. Each of the mutated versions of the enhancer sequence or the fragments can be introduced into an appropriate host cell and the efficiency of expression of a reporter gene measured. Those nucleotides required for enhancer function in this test are then identified as binding sites for specific proteins by means of gel mobility shift and DNA foot printing studies.

An alternate means of examining the capability of isolated fragments of the region upstream of the promoter to enhance expression of the reporter gene is to look for sub-domains of the upstream region that are able to enhance expression levels from a test promoter which comprises the TATA CAAT box but shows little or no detectable activity. A fragment of the 5'-region is inserted in front of

the test promoter in an expression cassette, and the effect on expression of the reporter gene evaluated.

Of particular interest for brain-specific, copy number-dependent expression are regions capable of binding to nuclear proteins in the region up to about 20kb from the mRNA start site of a brain-specific protein gene. Within this region, there may be several subdomains of interest having the characteristics of brain specific enhancer elements which can be evaluated by using constructs.

A promoter from a gene expressed in brain tissue of the host animal may be employed for varying the phenotype of the host animal. The transcriptional level should be sufficient to provide an amount of RNA capable of producing in a modified animal. By "modified animal" within the subject invention is meant an animal having a detectably different phenotype from a non-transformed animal of the same species, for example one not having the transcriptional cassette including APP coding sequences in its genome. Various changes in phenotype are of interest. These changes may include progressive neurologic disease in the cortico-limbic areas of the brain expressed within a short period of the time from birth; increased levels of expression of an APP gene above endogenous expression levels and the development of a neurologic illness accompanied by premature death; gliosis and intracellular APP/Aß accretions present in the hippocampus and cerebral cortex; progressive neurologic disease characterized by diminished exploratory/locomotor behavior and diminished 2-deoxyglucose uptake/utilization and hypertrophic gliosis in the cortico-limbic regions of the brain.

Of particular interest as a transcriptional initiation region is one from a prion protein gene which is functional in the brain of the host animal. Prion protein is implicated in the pathogenesis and transmission of Gerstmann-Straussler syndrome and in scrapie, an equivalent animal disease. Brain tissue serves as a source for nucleic acid for preparing the desired sequences. To identify a prion promoter having the desired characteristics, where a prion protein has been or is isolated, it may be partially sequenced, so that a probe may be designed for identifying mRNA specific for prion protein. Sequences which hybridize to the cDNA may then be isolated, manipulated, and the 5'untranslated region associated with the coding region isolated and used in expression constructs to identify the transcriptional activity of the 5'-untranslated region. As appropriate, sequences can

be amplified using PCR procedures known to those skilled in the art. In some instances, a probe may be employed directly for screening a genomic library and identifying sequences which hybridize to the probe. The sequences will be manipulated as described above to identify untranslated region. Prion promoter sequences are described in Basler, et al. (1986), *Cell* 46:417-428 and Scott, et al. (1992) *Protein Science* 1:986-987.

A variety of other promoter sequences can be used to control expression of APP coding sequences. These include the metallothionine (MT) promoter expression from which can be regulated through modulation of zinc and glucocorticoid hormone levels (Palmiter et al., *Nature* 300, 611-615 (1982)); the rat neuron specific enolase gene promoter (Forss-Petter et al., *Neuron* 5; 197-197 (1990)); the human ß actin gene promoter (Ray et al., *Genes and Development* (1991) 5:2265-2273); the human platelet derived growth factor B (PDGF-B) chain gene promoter (Sasahara et al, *Cell* (1991) 64: 217-227); the rat sodium channel gene promoter (Maue et al., *Neuron* (1990) 4:223-231); the human copper-zinc superoxide dismutase gene promoter (Ceballos-Picot et al., *Brain Res.* (1991) 552:198-214); and promoters for members of the mammalian POU-domian regulatory gene family (Xi et al., (1989) *Nature* 340:35-42). The POU-domain is the region of similarity between the four mammalian transcription factors Pit-1, Oct-1, Oct-2, and *unc*-86, and represents a portion of the DNA-binding domain. These promoters provide for expression specifically within the neurons of transgenic animals.

The termination region which is employed primarily will be one of convenience, since the termination regions appear to be relatively interchangeable. The termination region may be native with the transcriptional initiation region, may be native with the DNA sequence of interest, or may be derived from another source. Convenient termination regions are available from the prion protein gene.

The expression cassette which is used in the subject invention includes promoter and enhancer sequences from a gene which is expressed in the brain and preferably which is expressed in a manner that is related to the number of such sequences incorporated into the chromosome, namely that higher transcription occurs with a larger number of transgene copies incorporated into the chromosome, operably joined to an APP gene sequence and translational and

transcriptional termination regions. Examples of promoter and enhancer sequences which are expressed in brain and which drive copy number dependant expression include the prion protein promoter, such as that described by Scott et al . Protein Science (1992) 1:986-987, together with sequences upstream from the promoter, because in order to obtain copy number dependant expression, it generally is necessary to include a sufficiently large region of DNA controlling transcription so that it is large enough to be relatively unaffected by position effects. As an example, for the prion protein gene from hamster, approximately 20kb of sequence upstream of the promoter can be used.

As an example of construction of a cosmid vector for use in the instant invention, components which are assembled, in the 5' to 3' direction, include promoter and enhancer sequences of the prion protein gene, the coding region of an APP gene sequence of interest and transcriptional and translational termination sequences operably attached to a cosmid vector for delivery of the DNA constructs into the pronuclei of mouse eggs for expression of an APP gene in brain tissue. The enhancer sequences may include a 20 kb region upstream of the prion protein promoter and may also include the noncoding exon 1 and the 10 kb intron downstream of exon 1 from the prion protein gene or can include the coding sequence for more than one APP protein as described in, for example, W092/11276. Using molecular genetic techniques well known in the art, the promoter/enhancer region of the prion protein gene may be isolated from a mammalian genomic cosmid clone used to create transgenic mice which express prion protein. The coding sequence of an APP gene is inserted between the promoter/enhancer region and the termination sequences at a unique restriction site or sites such that the coding sequence is translated in-frame. An APP protein in transgenic brain tissue introduced using a cosmid vector as described above may be confirmed to be at least two to four-fold that of endogenous levels.

A major obstacle to the creation of a transgenic model of AD has been the inability to overexpress transgenic APP protein in the brain of the transgenic animal. In some cases, mRNA is well expressed, but the protein is poorly expressed. This indicates that the strength of promoters used may be adequate, but that protein translation may not be optimal. Poor translation may result from a weak translation initiation sequence. Accordingly, it may be necessary to include a

translation initiation sequence wherein the positions at minus three and plus four relative to the initiation codon are A and G, respectively. See Table 1 below.

Table 1

Transgene Translation Initiation Sequence Optimization

Transgene	Translation Initiation Sequence -3 +4
Hacos.CS0HuAPP695-V717Imyc	GCGATGCTG (native human APP)
Hacos.CS1	ACCATGCTG
Hacos. CS2	ACCATGGTG
Hacos.MoAPP695-WT	ACGATGCTG (native mouse APP)
Hacos.MoPrp-P101L	ATCATGGCG (native mouse PrP)

Any amyloid precursor protein sequence can be used to produce the transgenic animals of the invention. An APP protein sequence, as the term is used herein, means a sequence of the coding region of the APP gene which, when incorporated into the genome of the animal in multiple copies and expressed in the transgenic animal at supraendogenous levels, promotes a progressive neurologic disease in the transgenic animal. The neurologic disease is characterized by a neurobehavioral disorder with gliosis and diminished glucose uptake and/or utilization in cortico-limbic brain structures. The coding sequence can be from a wild-type gene, or from a gene containing one or more mutations. The coding sequence can be a natural sequence or a synthetic sequence or a combination of natural and synthetic sequences. By mutant is intended any APP which has an amino acid sequence which differs from that of the native APP and includes substitutions, deletions, and the like. By wild type APP is intended native APP as it occurs in the relevant host animal.

Native human APP is encoded by a single 400-kb gene comprised of 18 exons on chromosome 21. Alternative mRNA splicing gives rise to three APP isoforms. Two forms, APP-751 and APP-770 contain a Kunitz-protease inhibitor (KPI) region; the third, APP-695, lacks the KPI segment. Preferred sequences are those which are disease-linked. Examples of disease-linked mutations include a mutation at APP codon 693 (of APP-770) linked to Dutch congophilic angiopathy (Levy et al., (1990) *Science* 248:1124), a mutation in APP linked to familial AD, valine-isoleucine at codon 717 (of APP770) (Goate et al., (1991) *Nature* 349: 704-706), a mutation wherein the valine at codon 717 is replaced by phenylalanine or glycine (Chartier-Harlin et al., (1991) *Nature* 353: 844-846; Murrell et al., (1991) *Science* 254: 97-99); and in one family with both congophilic angiopathy and AD, a mutation wherein alanine is replaced by glycine at codon 692 (Hendriks et al., (1992) *Nature Genetics* 1:218-221). In a Swedish kindred, a double mutation at codons 670 and 671, resulting in a substitution of the normal lysine-methionine dipeptide by asparagine-leucine was found (Mullan et al ., (1992) *Nature Genetics* 1: 345-347). APP with M67ON-K67IL is reported to be associated with increased AB 1-40 secretion (Citron *et al.* (1992) *Nature* 360: 672-674; Cai et al. (1993) *Science* 259: 514-516), while enhanced AB 1-42 production is reported for APP with the V717I mutation (Cai et al. (1993), *supra*; Suzuki *et al.* (1994) *Science* 264: 1335-1340).

Table 2, below, lists some of the known amyloid precursor protein sequences, some of which are genetically linked to familial Alzheimer's disease.

Table 2[1]

Examples of APP Transgenes

Translation Initiation	APP ORF Species	ORF Size (codons)	Mutation
			V717I
			V717G
	human, mouse	695 &	V717F
CS1 or	or	751 or	VVM717/721/722IAV
CS2	human/mouse	770	MK670/671NL770
	chimeras		A692G
			E693Q

Of particular interest are novel chimeric APP genes, in which human Aß sequences replace the Aß region of mouse APP. A158,5 is a 4-kDa peptide derived from APP. Examination of 5 human (Hu), mouse (Mo), and chimeric (Mo/Hu) APP processing in mouse cell lines indicates that tangible differences are evident. It appears that HuAPP matures poorly in mouse cells, relative to Mo- or combination Mo/HuAPP. However, the human Aß sequences promote the formation of soluble Aß peptides that are normally produced. Mo/HuAPP chimeric protein matures more efficiently than HuAPP, and generates more soluble Aß than MoAPP.

Transgenic mammals are prepared in a number of ways. A transgenic organism is one that has an extra or exogenous fragment of DNA in its genome. In order to achieve stable inheritance of the extra or exogenous DNA fragment, the integration event must occur in a cell type that can give rise to functional germ cells, either sperm or oocytes. Two animal cell types that can form germ cells and into which DNA can be introduced readily are fertilized egg cells and embryonic stem cells.

[1]The abbreviations used in Table 2 refer to the following: CSl=translation initiation sequence as represented in FIG. 6; CS2=translation initiation sequence as represented in FIG. 7; V=valine; I=isoleucine; G=glycine; F=phenylalanine; M=methionine; A=alanine; X=lysine; N=arginine; L-leucine; E=glutamate; Q=glutamine; ORF=open reading frame; numeral in the 'Mutation' column refers to the mutated codon based upon the APP-770 numbering system.

Embryonic stem (ES) cells, can be returned from *in vitro* culture to a "host" embryo where they become incorporated into the developing animal and can give rise to transgenic cells in all tissues, including germ cells. The ES cells are transfected in culture and then the mutation is transmitted into the germline by injecting the cells into an embryo. The animals carrying mutated germ cells are then bred to produce transgenic offspring.

A preferred method for making the subject transgenic animals is by zygote injection. This method is described, for example, in USPN 4,736,866. The method involves injecting DNA into a fertilized egg, or zygote, and then allowing the egg to develop in a pseudo-pregnant mother. The zygote can be obtained using male and female animals of the same strain or from male and female animals of different strains. The transgenic animal that is born is called a founder, and it is bred to produce more animals with the same DNA insertion. In this method of making transgenic animals, the new DNA typically randomly integrates into the genome by a nonhomologous recombination event. One to many thousands of copies of the DNA may integrate at one site in the genome.

Generally, the DNA is injected into one of the pronuclei, usually the larger male pronucleus. The zygotes are then either transferred the same day, or cultured overnight to form 2-cell embryos and then transferred into the oviducts of pseudo-pregnant females. The animals born are screened for the presence of the desired integrated DNA. By a pseudo pregnant female is intended a female in estrous who has mated with a vasectomized male; she is competent to receive embryos but does not contain any fertilized eggs. Pseudo-pregnant females are important for making transgenic mice since they serve as the surrogate mothers for embryos that have been injected with DNA or embryonic stem cells.

Putative founders are screened for presence of the transgene in several ways. Brain APP protein and RNA expression are analyzed and the transgene copy number determined using methods known to those of skill in the art. Brain APP protein RNA expression, and transgene copy numbers are determined in weanling animals (4-5 weeks). Because the prion protein gene promoter is constitutionally active in animals of weanling age and older, it is not expected that there will be changes in levels of transgenic APP RNA expression animals beyond weanling age. APP levels can be monitored to determine whether there is a consistent

variation in expression levels with age. The transgenic animals also are observed for clinical changes. Examples of neurobehavioral disorders for evaluation are poor mating response, agitation, diminished exploratory behavior in a novel setting, inactivity, seizures and premature death. For transgene copy number, sufficient copies to achieve total brain APP expression from each construct from at least twofold, preferably at least two to fourfold, that of an endogenous native gene, is preferred. This number may range from five copies to more than 60 copies, depending on the species of APP expressed and the particular disease-associated mutations in the APP gene. Sufficient copies of a transgene therefore is that number which produces expression of APP at a level which results in a progressive neurologic disorder.

It is a theory of the invention that the clinical changes observed in transgenic mice are as a result of an increase in the amount of APP which is expressed, therefore sufficient copies of an APP gene are necessary to achieve a level of expression of particular APP gene which will result in observable clinical and/or behavioral symptoms, together with a measurable biochemical change in relevant brain structures. Less desirably, only biochemical changes would be obtained. By sufficient copies is intended that the total expression level from each construct is at least two-fold, preferably at least two to four-fold, that of an endogenous native gene, or that the overall copy number is such as to achieve this relative increase. In some instances, two to four copies of the gene, especially a mutated disease-linking gene, may be sufficient to achieve a desired relative increase in APP, while in others, particularly where a native gene is used, a larger copy number may be required. In some instances a lower amount of APP may be effective in producing a progressive neurologic disorder, particularly where the mutation in the APP occurs in the Aß region, or just upstream of the Aß region of the gene. The number of copies of a particular gene which are sufficient to obtain the desired result can be determined empirically. As an example, the effective range of copy numbers for HuAPP695.TRImyc is approximately 20 to 75; for HuAPP695.SWE is approximately 30 to 50; and MoAPP.wg is greater than 25.

The founder animals can be used to produce stable lines of transgenic animals that superexpress APP, either mutant or native APP. For ease of propagation, male founder mice are preferred. The animals are observed

clinically. Analyses of transgene copy number (to exclude multiple transgene insertion sites), mRNA expression, protein expression, neuropathology, and glucose uptake in these animals are also performed. These studies provide information about the age of onset of illness, the duration of illness, the penetrance of the phenotype, the range of neuropathologic 5 findings, regional brain dysfunction, and the dependence of phenotype upon levels of protein expression.

The animals also are screened using a species appropriate neurobehavioral test. For example, studies of locomotor/exploratory behavior in mice is a standard means of assessing the neuropsychology (File and Wardill, (1975) *Psychopharmacologia* (Berl) 44:53-59; Loggi et al., (1991) *Pharmacol. Biochem. Behav.* 38:817-822). For example, for mice the "corner index" (CI) is used. This is a quick and simple neurobehavioral test to screen animals for evidence of brain pathology. The CI in transgenic mice which express mutant and wild-type APP is also measured. A low CI (≤ 4) correlates with high mutant APP transgene copy numbers, premature death, and neuropathologic findings. The CI exhibits a dosage dependent relationship to transgene copy number, which supports the validity of its use in assessing neurobehavioral signs in transgenic mice.

The neuropathology of the animals also is evaluated. Brain regions known to be affected by AD, such as those in the cortico-limbic region, are particularly reviewed for changes including APP/Aß excretions, gliosis, and changes in glucose uptake and utilization. Immunohistologic studies of various brain regions is used to detect transgene product.

The animals used as a source of fertilized eggs cells or embryonic stem cells, the "host animal", can be any animal, although generally the preferred host animal is one which lends itself to multigenerational studies. Of particular interest are rodents including mice, such as mice of the FVB strain and crossed of commercially available strains such as the (C57B6) x (SJL.F1) hybrid and the (Swiss Webster) x (C57B16/DBA-z.F1) hybrid. The latter parental line also is referred to as C57B16/D2. Other strains and cross-strains of animals can be evaluated using the techniques described herein for suitability for use as a model for progressive neurologic diseases such as AD. In some instances, however, a primate, for example a rhesus monkey may be desirable as the host animal, particularly for therapeutic testing.

The animals of the invention can be used as tester animals for materials of interest, *e.g.* antioxidants such as Vitamin E or lazaroids, thought to confer protection against the development of AD. An animal is treated with the material of interest, and a reduced incidence or delayed onset of neurologic disease, as compared to untreated animals, is detected as an indication of protection. The animals further can be used as tester animals for materials of interest thought to improve or cure Alzheimer's disease. An animal with neurologic disease is treated with the material of interest, and a delayed death, or improvement in neurobehavior, gliosis, or glucose uptake/utilization, as compared to untreated animals with neurologic disease, is detected as an indication of amelioration or cure.

The animals of the invention can be used to test a material or situation, e.g. oxidants or head trauma, suspected of accelerating or provoking Alzheimer's disease, by exposing the animal to the material or situation and determining neurobehavioral decline, premature death, gliosis, and diminished glucose uptake/utilization as indicators of the capacity of the test material or situation to induce Alzheimer's disease. The method further can include testing of therapeutic agents by exposing animals to a material or situation suspected of provoking Alzheimer's disease and evaluating the effect of the therapeutic agent.

Careful characterization of the transgenic animals should lead to elucidation of the pathogenesis of AD. The sequence of molecular events in mutant APP metabolism leading to disease can be studied. The animals also are useful for studying various proposed mechanisms of pathogenesis, including horizontal transmission of disease (Prusiner, et al. (1987) *Cell* 63, 673-86), oxidation and free-radical production (Blass and Gibson, (1991) *Rev. Neurol* (Paris) 147:513-525; Ames et al., (1993) *Proc. Nat'l. Acad. Sci. USA* 90:7915-7922), inflammation (McGeer et al., (1993) *Can. J. Neurol. Sci.* 18:376-379, Rogers et al., (1992) *Proc. Nat'l. Acad. Sci. USA* 89:10016-10020); neurotrophic factor deprivation (Perry, (1990) *Alzheimer's Disease and Associated Disorders* 4:1-13; Hefti and Schneider, (1991) *Clinical Neuropharmacology* 1:62-76); Koliatsoess et al., (1991) *Ann. Neurol.* 30:831-840), apolipoprotein E4 metabolism (Strittmatter et al., (1993) *Proc. Nat'l. Acad. Sci. U.S.A.* 90:1977-1981), and potassium channel dysfunction (Etcheberrigaray, et al., (1993) *Proc. Nat'l. Acad. Sci. USA*

90:8209-8213). Such knowledge would lead to better forms of treatment for neurologic disorders.

Other features and advantages of the invention will be apparent from the description of the preferred embodiments, and from the claims. The following examples are offered by way of illustration and not by way of limitation.

EXAMPLES

Examples 1

PrP-HuAPP Transgene Construction.

The human APP coding sequence was derived from a human cDNA (see Kang et al. (1987) *Nature* 325:733; Goldgabar et al. (1987); *Science* 235:877; Tanzi et al. (1987); *Science* 235:880; and Robakis et al. (1987) *Proc.Nat.Acad.Sci.* (USA) 84:4190) and is illustrated in FIG. 1. It occurs in three splice forms which are derived from a gene located on chromosome 21 as described by Kitaguchi et al. (1988) *Nature* 331:530; Tanzi et al. (1988) *Nature* 331:528; and Ponte et al. (1988) *Nature* 331:525. FIG. 2 illustrates three features which may be incorporated into amyloid precursor protein sequences to produce the transgenic animals of the invention: (1) splice form variants which result from the presence or absence of the Kunitz protease inhibitor with or without the OX region; (2) amyloid precursor protein variants containing mutations which have been linked to illness in families with Alzheimer's disease as described by Goate (1991) *Nature* 349:704; Chartier-Harlin et al. (1991) *Nature* 353:844; Murrell et al. (1991) *Science* 254:97; Hendriks et al. (1992) *Nature Genetics* 1:218; and Mullan et al. (1992) *Nature Genetics* 1:345, and families with congophilic angiopathy as described by Levy et al. (1990) *Science* 248:1124, and (3) a myc-tag at the carboxyl terminus which can be used to facilitate immunodetection of transgene products, but is preferably absent.

The required hamster prion protein gene functions were provided by a hamster prion protein cosmid vector in which a tetracycline-resistance sequence flanked by *Sal*I sites replaces the prion protein coding sequence, as described by Scott et al. (1992) *Protein Science* 1: 986. The hamster prion protein cosmid vector is illustrated in FIG. 3. A 1.6 KB region of DNA in the 3'-untranslated

region of the prion protein gene is indicated as a useful probe for detecting transgenes made from this cosmid.

The APP sequences and cosmid were used to construct the two fusion gene constructions illustrated in FIGS. 4 and 5. The APP sequences were modified for strong translation initiation, represented by the abbreviations CS1 and CS2. The constructions were made by substituting the *SalI* to *Kpn*I DNA sequence at the 5' end of the APP coding sequence for DNA sequences made using the polymerase chain reaction (PCR) and two sets of primers. For the CS1 APP sequence illustrated in FIG. 6, the primer set used was 5'-AAGTCGACACCATGCT GCCCGGTTTGGCACT-3' and 5'-AAGGTACCTCCCAGCGCCCGAGCC-3'. For the CS2 APP sequence illustrated in FIG. 7, the primer set used was S'-AAAAAAGTCGACACCATGGTGCCCGGTTTGGCACT-3' and 5'-AAGGTACCTCCAGCGCCCGAGCC-3'.

Procedures were the conventional techniques described in Maniatis et al. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory) and the polymerase chain reaction (PCR) described in Saiki et al. (1988) *Science* 239:487. The restriction sites shown in FIGS. 1-7 are *SalI* (S), *Kpn*I (K), *Bgl*II (B), *Xho*I (X) and *Not*I (N). The location of the PCR oligomers used for detecting fusion constructs in animals are indicated by A and P in FIG. 8. Each PCR fragment synthesized for the constructions was sequenced. The PCR fragments selected for use in the constructions were free of unintended mutations.

The above PrP-APP cosmids were digested with *Not*I which releases the PrP-APP fusion gene from the pcos6EMBL vector illustrated in FIGS. 3-5. The PrP-APP fusion gene was isolated after size fractionation on an agarose gel and electroeluted. The PrP-APP fusion gene was further purified in a series of organic extractions, including phenol-chloroform, chloroform, and butanol, and precipitated in ammonium acetate and ethanol. Prior to embryoinjection, the PrPAPP fusion gene was dissolved in 10mM Tris-C1 (pH 8.0) to a final concentration of 3-4 μg/ml.

Example 2

Production of Transgenic Mice
Containing PrP-HuAPP Transgene
(APP Sequence VM717/721/722IAV)

Each PrP-APP fusion gene was separately injected into fertilized one-cell mouse eggs (Hogan *et al.* (1986) *Manipulating the Mouse Embyro: A Laboratory Manual,* Cold Spring Harbor Press, N.Y.; also see USPN 4,736,866). Embryo donors and fertile studs were inbred FVB mice obtained from the National Cancer Institute (NIH); this resulted in the integration of between 1 and 128 copies of PrP-APP fusion genes into the genomes of the mice which developed to term. The injected eggs were transferred to pseudo-pregnant foster females as described in Wagner et al. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78:5016.* Mice were housed in an environmentally controlled facility maintained on a 10 hour dark: 14 hour light cycle. The eggs in the foster females were allowed to develop to term.

Example 3

Analysis of VVM717/721/722IAV Transgenic Mice

At four weeks of age, each pup born was analyzed in a PCR reaction using DNA taken from the tail. In each case, tail DNA was used as a template for a PCR reaction using the probes indicated in FIG. 8. The DNA for analysis was extracted from the tail by the method described in Hanley and Merlie (1991) *Biotechniques* 10:56. 1 ml of the tail DNA preparation (approximately 1 mg of DNA) was used to amplify a transgene specific DNA fragment in a 25Z1 PCR reaction containing primers A and P as illustrated in FIG. 8.

The PCR reactions indicated that 15 founder mice had retained an injected PrP-APP fusion gene. The APP sequence in these animals contained the VVM717/721/722IAV mutation and the myc-tag, but lacked the KPI/OX regions represented in FIG. 2. To determine transgene copy number, denatured DNA in an exponentially diluted series was probed with a 1.6 kilobase (KB) radiolabelled segment of DNA from the 3'-untranslated region of the hamster PrP gene as illustrated in FIG. 3. Among the founder mice with the highest transgene copy numbers (approximately 100 or more), two founder mice failed to breed, and a third founder sired offspring, which in turn failed to breed. Thus, the 15 founder

mice yielded 12 lines of transgenic offspring. A catalog of transgenic founders with APP transgenes is shown in Table III.

The founder animals were mated to uninjected animals and the DNA of the resulting 12 lines of transgenic offspring analyzed: this analysis indicated that in every case the injected genes were transmitted through the germline.

Table III

Catalog of Transgenic Founders with APP Transgenes

Animal ID	Transgene	Transgene Copy #	Protein Level	Status
Tg425L	Hacos.CSOHuApp695-V717Imyc	1	Not detectable	Sac'd
Tg466M	Hacos.CSOHuApp695-WTmyc	32-64	1.5-2X	Alive
Tg1056L	Hacos.CS1HuApp695-V717Imyc	16		Alive
Tg1057H	Hacos.CS1HuApp695-V717Imyc	64-128		Dead
Tg1064L	Hacos.CS1HuApp695-V717Imyc	8		Alive
Tg1072L	Hacos.CS2HuApp695-V717Imyc	1		Alive
Tg1073L	Hacos.CS2HuApp695-V717Imyc	1		Alive
Tg1118M	Hacos.CS1HuApp695-V717Imyc	32-64		Alive
Tg1119L	Hacos.CS1HuApp695-V717Imyc	1		Alive
Tg1123L	Hacos.CS1HuApp695-V717Imyc	1		Alive
Tg1125L	Hacos.CS1HuApp695-V717Imyc	8-16		Alive
Tg1130H	Hacos.CS1HuApp695-V717Imyc	64-128		Sick
Tg1135H	Hacos.CS2HuApp695-V717Imyc	64-128		Dead
Tg1138H	Hacos.CS2HuApp695-V717Imyc	64-128		Dead
Tg1140M	Hacos.CS2HuApp695-V717Imyc	32-64		Alive

Six founder animals harbored >20 copies of the PrP-APP fusion genes. All six developed a neurologic disease characterized by progressively diminishing exploratory/locomotor behavior and premature death by five months of age. In contrast, none of nine founder animals harboring <20 copies of the PrP-APP fusion genes have developed the neurologic disease within the first five months of age. The neurologic dysfunction was transmitted to succeeding generations in an autosomal dominant fashion.

Expression of the newly acquired PrP-APP fusion genes in tissues was determined by Western blot analysis using a monoclonal antibody, 6E10, raised to the first 17 residues of the human Aß peptide (Kim, et al. (1990) *Neuroscience Research Communicating* 7:113-122). The fusion gene product was detected in the brain, spinal cord, skeletal muscle, heart, and, minimally, lung. It was not detected in the liver, spleen, kidney, or testis.

Expression of the PrP-APP fusion gene in brain tissue was quantitated by immunodot blot analysis. Relative APP expression in brain tissue was compared in transgenic and non-transgenic mice in an exponentially diluted series and reacted with antibody recognizing the 15 residues at the carboxyl terminus of APP, CT15, which recognizes both mouse and human APP (Sisodia, et al. (1993) *J. Neurosciences* 13:3136-3142). The total APP protein in lines of mice which developed the neurologic disease was at least 300% of endogenous levels. Where expression was less than 300%, animals did not develop neurologic disease.

To obtain an index of brain function in affected transgenic mice, glucose utilization was regionally determined using a modification of the Sokoloff method described by Chmielowska et al. (1986) *Exp. Brain Res.* 63: 607, which allows glucose uptake/metabolism in the mouse to be measured. Regional 2-deoxyglucose concentrations determined densitometrically were normalized to the cerebellum, a region devoid of pathology. Results in transgenic mice revealed significant reductions in glucose utilization of 20-30% in the hippocampus, amygdala, and some regions of the cerebral cortex as compared to age-matched non-transgenic littermates.

Example 4

Analysis of Synthesis and Processing In Vitro

The synthesis and processing of the VVM717/721/722IAV mutant in cultured cells was examined to determine the effects of these mutations on disease development. The wild-type HuAPP695myc and mutant cDNA genes were cloned into the expression vector pEF-BOS (Osaka Bioscience Institute, Osaka, Japan), then transiently transfected into mouse neuroblastoma cells, which were then continuously labeled with [^{35}S]methionine for 4 hours. Labeled APP molecules were immunoprecipitated with the monoclonal antibody 22C11 (Weidemann, et al. (1989) *Cells* 57:115-126). In extracts of cells, labeled APP molecules of the

appropriated size were detected in similar levels. Media from these cultures was examined for the presence of soluble APP fragments using mAb 6E10 and mAb 4G8 (Kim, et al. (1990) *supra.*). Both of these antibodies recognize the Aß region of human APP. The mAb 6E10 recognizes sequences in Aσ between Aß 1-17, while mAb 4G8 recognizes sequences between Aßl-28. The sequence of Aß17-28 is identical to mouse Aß and thus 4G8 cannot distinguish human and mouse APP. The media of cultures transfected with either gene contained a large ectodomain fragment of APP which is routinely observed.

One of the more recent discoveries relevant to the processing of APP has been the detection of soluble Aß 1-40 fragments in the medium of cultured cells that express HuAPP. These Aß fragments resemble peptides found in AD amyloid plaque lesions. Thus, it appears that APP is normally processed into amyloidogenic fragments. Furthermore, mutations linked to AD have been shown to alter the processing of APP to favor the production of soluble Aß. To determine whether the VVM717/721/722IAV mutations affected the processing of APP, the culture medium was examined for small Aß-containing APP peptides. An Aσ peptide fragment that was immunopurified by mAb 6E10 was prevalent in the media of cells transfected with the mutant sequence. Similarly, the mAb 4G8 detected increased levels of Aß peptide in the medium of cultures containing the mutant.

An examination of cell extracts for accumulated APP fragments detected increased levels of a 10 kDa APP peptide fragment after immunoprecipitation with anti-myc polyclonal antiserum in cells expressing the mutant (Fig. 5C, line 3). Mutations generated in mutant HuAPP695myc affect the processing of the resultant APP product to generate increased levels of soluble Aß, and an intracellular C-terminal fragment of APP that is of sufficient length to include the Aß region. Thus, the phenotype of animals created with the mutant APP is much like that reported for humans expressing a mutant human APP gene that encodes mutations found in a Swedish kindred of AD. To date no investigators have reported increased production of Aß as a result of expression of HuAPP that encodes only the V642I AD-linked mutation (Golde et al., (1993), *Neuroscience*

Abstract 19:431, 182.7). However, this mutation appears to cause a change in the length of the soluble Aß derivative, increasing it to Aß1-42. Thus is appears that the VVM717/721/722IAV mutations are the primary cause of the increased production of soluble Aß. Studies on Aß fibrillogenesis suggest that longer Aß peptides are more amyloidogenic.

Example 5

Comparison of the Processing of Human and Mouse APP in Mouse Cells

Chimeric APP transgenes composed of mouse APP695 and human Aß sequences were prepared and their processing evaluated. It is an hypothesis of the invention that there are differences in the way mouse and human APP are processed in mice. To construct humanized MoAPP cDNA, a MoAPP gene was cloned and mutated to make it compatible with the cosSHaPrP.535 vector. Mouse cDNA was isolated by reverse transcriptase-polymerase chain reaction (RTPCR), and PCR primers included *XhoI* sites at the 5' and 3' ends for cloning purposes. To remove an internal *XhoI* site in the mouse cDNA, an additional primer was included that spanned the internal *XhoI* site (codon 397) and contained a single base substitution that eliminated the *XhoI* site but preserved the correct amino acid sequence. The PCR product was subsequently sequenced to verify that unwanted mutations were not created in the PCR.

The Aß region in HuAPP and MoAPP differs by three amino acid residues, which could affect the amyloidogenic potential of the transgene product. To humanize the mouse Aß region, a segment of the HuAPP gene that encompassed the Aß region was amplified by PCR using primers that include a sense primer that encompassed the *Bgl*II site at codon 590 of HuAPP-695 and an antisense primer that contained two point mutations creating a *Nar*I site at codon 626 (a cognate *Nar*I site is found in the MoAPP cDNA), while maintaining the amino acid sequence (Table 4, primers 1 and 2). This PCR product was digested with *Bgl*II and *Nar*I and then cloned into the *Bgl*II and *Nar*I sites of the MoAPP cDNA.

The chimeric (Mo/HuAPP) cDNA was sequenced across the *Bgl*II and *Nar*I sites to verify that this region now contained human Aß sequences and to verify that no other unwanted mutations were generated. To verify that this recombinant cDNA could be expressed into full-length protein, DNA was cloned into a modified pEFBOS vector. The pEF-BOS vector contains the promoter element, first exon, first intron, and part of the second exon of the mammalian elongation factor 2α along with an SV40 origin of replication, permitting the replication of vectors and the high expression of genes in COS-1 cells. COS-1 cells were transfected with pEF-BOSMo/HuAPP-695 and cell extracts were analyzed by immunoblotting. CT15 recognized a full-length Mo/HuAPP polypeptide, whereas immunostaining with monoclonal antibody 6E10 verified that the humanized mouse cDNA product did indeed encode human Aß sequences.

To generate chimeric Mo/HuAPP cDNA that encodes a double mutation linked to an early-onset AD, a PCR-based approach similar to that outlined above using primers 2 and 3 (Table 4) was employed. The template for the reactions was a cloned copy of Mo/HuAPP-695. The mutated chimeric gene was sequenced across the *Bgl*II and *Nar*I sites to verify the presence of mutations and to be certain that no unwanted mutations existed in the transgene. The mutant Mo/HuAPP cDNA was cloned into pEFBOS and transfected into COS-1 cells to determine whether APP polypeptides were synthesized. An APP polypeptide of the predicted size reacted with both CT15 and 6E10 antibodies.

An examination of the synthesis and processing of Mo-, Hu-, and Mo/HuAPP in mouse N2a cells has surprisingly revealed discernible differences. What is evident is that a greater percentage of MoAPP is cleaved to generate a soluble ectodomain fragment than is HuAPP. The ratio of cell-associated versus soluble moAPP is approximately 1 to 5, while 3 times more of the HuAPP is cell-associated than is soluble. The percentage of Mo/HuAPP695 that is cleaved to generate soluble ectofragments appears to fall between that of Mo- and HuAPP as the ratio of cell-associated to soluble Mo/HuAPP approaches 1 to 1. The majority of soluble APP ectofragments appear to arise from a cleavage event within Ad at the cell surface; the differences in the ratio of cell-associated APP versus soluble

ecto-fragments indicate differences in the maturation of the polypeptides. Specifically, the majority of MoAPP reaches the cell surface and is cleaved by a secretase. In contrast, HuAPP may not reach the cell surface as efficiently, thus precluding secretase cleavage. The Mo/HuAPP polypeptide appears to be intermediate between Mo and HuAPP. Alternatively, it is possible that sequences within the Aß domain influence the efficiency of secretase cleavage.

In addition to differences in the production of soluble APP ecto-fragments, differences in the level of soluble Aß peptides were noted. All three proteins gave rise to soluble Aß peptides that were of a size and character consistent with identification as Aß1-40. In cells transfected with MoAPP, a fragment that is of a size and character consistent with identification as Aß17-40 was detected. The Aß17-40 fragment is thought to arise after membranal cleavage of APP by the putative asecretase, which cleaves between Aß16 and -17. Only the Hu- and MoHuAPP derived Aß1-40 peptides were recognized by mAb6E10 as expected. While MoAPP appeared to give rise to relatively equal amounts of Aß1-40 and Aß17-40, HuAPP and Mo/HuAPP were preferentially cleaved to generate only Aß1-40. These results suggest that sequences differences within the human Aß domain influence APP proteolytic cleavage.

Example 6

Comparison of Normal Aged Mice and Transgenic Mice

Transgene Construction.

The PrP-APP transgenes were generated as described in Example 1 by replacing a *Sal*I-flanked tetracycline resistance sequence in a hamster PrP cosmid vector (Scott *et al*, (1992), *supra*), with *Sal*I-flanked human and mouse APP coding sequences. Tg mice were prepared using one of six different PrP/APP chimeric transgenes: murine wild-type APP695 (MoAPP695.WT); human APP-695 containing two mutations at M670N and K671L (APP770 numbering) (HuAPP695.SWE); human APP695 containing two mutation at M670N and K671L (APP770 numbering) (HuAPP695.SWE); human APP695 containing a mutation at E693Q (HuAPP695.DUT); human APP770 with M670N and K671L

(HuAPP770.SWE); human APP695 with a triple mutation at V717I, V7211A, and M722V with a 3'-myc tag (HuApp695.TRImyc); and human wild-type APP695 with a 3'-myc tag (HuApp695.WTmyc). The CS1HuAPP695.SWE, CS1HuAPP770.SWE, CS1HUAPP695.TRImyc and CS2HuApp695.TRImyc APP sequences were modified for strong translation initiation.

Like the Swedish mutation, triple V7171I, V721A and M722V mutations in the transmembrane domain of APP enhance secretaion of Aß by five-fold in cultured cells. The 3'-myc tag, a 12 codon segment of the c-myc proto-oncogene, was shown in cultured cells to facilitate immunodetection of transfection products (Wong and Cleveland, (1990) *The Journal of Cell Biology* 111, 1987-2003). In Tg(HuAPP695.WTmyc) and Tg(HuAPP695.TRImyc) mice the myc-tag was not as clearly detectable in Western blots and histologic samples as HuAPP reacted with human-specific APP antibodies. The myc-tag exerted no apparent effect on the phenotype, since Tg(HuAPP695.SWE),T g(HuAPP770.SWE), and Tg(HuAPP695.DUT) mice lacking the myc-tag develop the same clinical and pathologic abnormalities. The constructions were made by substituting the *Sal*I to *Kpn*I DNA sequence at the 5' end of the APP coding sequence for DNA sequences made using the polymerase chain reaction(PCR) and two sets of primers. For the CS1 APP sequence, the primer set used was 5'-AAGTCGACACCATGCTGCCC-GGTTTGGCACT-3' and 5'-AAGGTACCTCCCAGCGCCCGAGCC-3'. For the CS2 APP sequence, the primer set used was 5'-AAAAAAGTCGACACCATGGT-GCCCGGTTTGGCACT-3' and 5'-AAGGTACCTCCAGCGCCCGAGCC-3'. The HuAPP mutations were made using standard methods of site-directed mutagenesis. Each PCR fragment synthesized for the constructions was sequenced. The PCR fragments selected for use in the constructions were free of unintended mutations. The PrP-APP cosmids were digested with *Not*I (which releases the PrP-APP fusion gene from the pcos6EMBL vector). The PrP-APP fusion genes were isolated after size fractionation on an agarose gel and electroeluted. The PrP-APP fusion gene was further purified with organic solvents, and precipitated in ammonium acetate and ethanol. The PrP-APP fusion genes were dissolved in 10mM Tris-Cl (pH 8.0) to a final concentration of 3-4 μg/ml prior to embryo injection.

1503: 5'-CTGACCACTCGACCAGGTTCTGGGT-3' and 1502: 5'-GTGGATAA-CCCCTCCCCCAGCCTAGACCA-3', located in the 3' region of APP and the 3'-untranslated region of PrP, respectively. The 1503 primer recognizes a region which is homologous in mouse and human APP, and can therefore be used to detect both PrP-MoAPP and PrP-HuAPP DNA. Using primers 1502 and 1501: 5'-AAGCGGCCAAAGCCTGGAGGGTGGAACA-3', a parallel PCT reaction amplifying a fragment of murine PrP was performed as a positive control.

Transgene copy number analysis was performed using 5μg denatured purified tail DNA baked onto nitrocellulose and hybridized to a radiolabelled 1.3kb *SalI-XhoI* DNA fragment encoding a segment of the hamster PrP 3'-untranslated region located in the DNA sequence at the 5' end of the APP coding sequence for DNA sequences made using the polymerase chain reaction (PCR) and the two sets of primers described in Example 1. The HuAPP mutation were made using standard methods of site-directed mutagenesis. Each PCR fragment synthesized for the constructions was sequenced. The PCR fragments selected for use in the construction were free of unintended mutations. The PrP-APP cosmids were digested with *NotI* and the PrP-APP fusion genes were isolated after size fractionation on an agarose gel and electroeluted and further purifies as described in Example 1. The PrP-APP fusion genes were dissolved in 10mM Tris-Cl (pH8.0) to a final concentration of 3-4 μg/ml prior to embryo injection.

Transgenic Mouse generation and screening

Transgenic lines were initiated by microinjection of single-cell mouse embryos as described (Hogan et al., (1986) *supra*). Embryo donors and fertile studs were inbred FVB mice obtained from the National Cancer Institute (NIH). Post-weaning tail biopsy DNA was generated as described (Hanley and Merlie, (1991) *Biotechniques* 10, 56). One microliter of the unpurified DNA was used in a 25μl PCR reaction. To detect PrP-APP fusion DNA, the PrP-APP fusion DNA was amplified using the polymerase chain reaction with a pair of oligomer primers, 1503: 5'-CTGACCACTCGACCAGGTTCTGGGT-3' and 1502: 5'-GTG-GATAACCCCTCCCCCAGCCTAGACCA-3', located in the 3' region of APP and the 3'-untranslated region of PrP, respectively. The 1503 primer recognizes a

region which is homologous in mouse and human APP, and could therefore be used to detect both PrP-MoAPP and PrP-HuAPP DNA. Using primers 1502 and 1501: 5'-AAGCGGCCAAAGCCTGGAGGGTGGAACA-3', a parallel PCR reaction amplifying a fragment of murine PrP was performed as a positive control.

Transgene copy number analysis was performed using 5 μg denatured purified tail DNA baked onto nitrocellulose and hybridized to a radiolabelled 1.3kb *SalI-XhoI* DNA fragment encoding a segment of the hamster PrP 3'-untranslated region located in the hamster PrP cosmid vector (Scott, et al., (1992) supra). After two high-stringency washes and exposure to radiosensitive film, the relative intensities of signals from genomic DNAs of transgenic mice and hamsters were compared using a phosphorimager to obtain transgene copy numbers relative to diploid hamster genomic DNA.

Analysis of transgene expression

APP transgene product expression was examined in progeny of transgenic founders sacrificed at one to four months of age. Quantitative immunoblotting of extracts from brain homogenates was carried out in parallel with extract prepared from age-matched nontransgenic littermates. 20% (w/v) homogenates of brain tissues were prepared in TNE (50mM Tris-Cl Ph 8.0, 150 mM Na Cl, 5 mM EDTA with 2% PMSF) buffer, using a hand-held Polytron. Homogenates were diluted with an equal volume of TNE 1% N40, 1% Deoxycholate, 0.4% SDS and sonicated in a bath sonicator until all viscosity was lost. Homogenates were then boiled for 10 minutes and centrifuged at 10,000 x g for 10 minutes.

The supernatants were mixed with an equal volume of 2 X sample buffer (Laemmli, (1970) *Nature* 227, 680-685), boiled 2 min, and fractionated using a 6% SDS-PAGE. Proteins were electrophoretically transferred to Immobilon membranes (Pierce) and incubated with polyclonal (CT15 and antiGID) and monoclonal (22C11 and 6E10) APP antibodies. Reactive rabbit polyclonal antibodies were visualized following incubation with secondary rabbit antibodies to mouse IgG before incubation with ^{125}I-protein A. Radiointensities were quantified on a phosphorimager (Molecular Dynamics, Inc.). APP expression in brain tissue was measured in Tg mice harboring different transgene copy numbers by

quantification of immunoblots in Tg lines with three antibodies recognizing both MoAPP and HuAPP, CT15 (Figure 11), anti-GID (Figure 11), and 22C11 (Figure 11). CT15 (Sisodia et al., (1993) *J. Neurosciences* 13:3136-3142; Borchelt et al., (1994) *J.Biol.Chem* 269: 14711-14714); anti-GID (Cole et al., (1989) *Brain Res. Reviews* 13:325-349); and 22C11 (Weidemann et al., (1989) *Cell* 57:115-126) recognize both mouse and human APP equally, but 22C11 also binds APLP2, a close relative of APP, with the same avidity (Slunt et al., (1994) *J.Biol.Chem* 269:2637-2644). Minor variations in HuAPP levels relative to MoAPP expression obtained with different antibodies may reflect differences in the avidity of antibody binding or distinctions in post-translational processing between wild-type and variant HuAPP. Transgenic brain APP protein expression was dependent upon copy number as well as the species of APP expressed (Figure 12). Relative to HuAPP, equivalent levels of MoAPP were achieved with lower numbers of transgene copies.

To measure the level of HuAPP specifically, brain homogenates were probed with 6E10 antibody raised against residues 1-17 of human Aß (Kim et al., (1990) *Neuroscience Res. Comm.* 7:113-122). No reactivity to ~ 100-125 kD APP molecules was detected in non-Tg mice (Figure 11). In Tg1130H mice the highest levels of HuAPP detected on immunoblots using 6E10 antibody were in the brain and spinal cord, and much smaller amounts (<5% of brain levels) were found in the striated muscle, heart, skin, and lung. HuAPP was poorly detected or absent in the thymus, liver, spleen, kidney, testis, and small intestine.

Specific immunostaining for human APP/Aß using the 6E10 or 8E5 antibody (*Athena Neurosciences*) revealed HuAPP throughout the brain. 8E5 recognizes a segment of APP spanning residues 444-592 (APP695 numbering). Two different methods were used to enhance APP immunoreactivity in brain tissue from Tg lines overexpressing HuAPP. In high copy number lines, following either formic acid pretreatment of tissue using 1:5000 dilution of 6E10 antibody or microwave pretreatment of tissue using either 1:100 6E10 antibody or 1:100 8E5 antibody, APP staining was invariably present within vesicular structures in large pyramidal cells of the hippocampus, parahippocampal area, amygdala, and the

cerebral cortex (Figure 13A,C,H). In some brains, fainter immunoreactivity was also visible in smaller neurons in the cortico-limbic regions of the brain and in large and small neurons of the basal ganglia, brainstem, and cerebellum. Staining was absent in non-Tg mice (Figure 13B,H) and in untreated brain tissue from affected Tg mice. The pattern of HuAPP immunostaining obtained reflected the widespread expression of HuAPP in the brain with the highest levels of expression in the telencephalon, as independently confirmed in regional brain immunoblots using the 6E10 antibody (Figure 11).

The 8E5 antibody stained amyloid plaques and intraneuronal vesicular structures in microwaved tissue sections from patients with AD (Figure 13F). At 1:5000 dilution, the 6E10 antibody stained amyloid plaques from patients with AD only after formic acid pretreatment of brain tissue (Figure 13D,E). However, in TgHuAPP mice neither the microwave nor formic acid pretreatment of brain tissue revealed HuAPP staining resembling extracellular amyloid or pre-amyloid deposits using either antibody. The abnormal phenotype in these Tg mice, therefore, was not caused by amyloid or pre-amyloid deposition.

To assess the relative effects of mutant and wild-type APP transgene expression on the development of a CNS disorder, the percentage of animals sick or dead at 100 and 200 days in lines expressing different levels of wild-type HuAPP, mutant HuAPP, or wild-type MoAPP (Table 5) was determined. These data demonstrate a direct relationship between APP expression and the development of an abnormal phenotype (Figure 15). A comparison of Tg mice expressing wild-type HuAPP and mutant HuAPP was not possible over the full range of APP expression. However, a comparison of TG mice expression approximately two to four fold mutant HuAPP, (TgHuAPP695.TRImyc)1140 and (TgHuAPP695.TRImyc)1130, with Tg mice expressing approximately three fold wild-type MoAPP, (TgMoAPP695.WT)1874, indicates that mutant HuAPP will readily provokes the abnormal phenotype. This observation argues against the abnormal phenotype being due to a non-specific effect of Tg protein over expression, since mutant HuAPP conferred the disorder with higher penetrance than wild-type MoAPP, demonstrating a specific effect of the Tg protein species it

expressed. These data are represented as titration curves that demonstrate a direct relationship between APP expression and the development of an abnormal phenotype (see Figure 15). However, the left-shifted curve for Tg mice expressing mutant APP relative to wild-type APP indicates that expression of the mutant APP more readily provokes the abnormal phenotype.

To ensure that overexpression of a foreign (human) species of protein did not artefactually produce the abnormal phenotype, Tg mice overexpressing wild-type MoAPP were generated. In Tg mice with MoAPP levels equivalent to 3.1-fold endogenous APP levels the same phenotype occurred, indicating that the observed phenotype was not due to overexpression of a foreign species of protein.

Table 5.

Clinical and pathological features of FVB mice expressing APP transgenes

Line	Copy Number (mean±SEM)	Transgenic brain APP (mean±SEM)	% sick or dead at 100 days	% sick or dead at 200 days	Cortico-limbic gliosis (in affected mice)	Extracellular HuAPP deposits	Weight (gm±SEM)	
							Tg	Non-Tg
Tg(HuAPP695.TRImyc)1072		<0.05	0 (n=21)	0 (n=21)				
Tg(HuAPP695.TRImyc)1056	7±1.7	0.3±0.09	0(n-10)	10 (1/10)				
Tg(HuAPP695.TRImyc)1118	21±3.7	1.4±0.17	42.5 (20/47)	97 (20/30)	+ (1/1)	-	21.9±0.46	
Tg(HuAPP695.TRImyc)1140	49±2.5	2.0±0.32	83 (15/18)	93 (15/16)			18.0±0.92	20.6±0.38
Tg(HuAPP965.TRImyc)1130H	74±3.7	3.6±0.54	98 (59/60)	100 (60/50)	+ (3/3)	-	11.4±0.50	
Tg(HuAPP695.TRImyc)1057F	64-128	NA	100 (1/1)	100 (1/1)	+ (1/1)	-		
Tg(HuAPP695.TRImyc)1138	64-128	NA	100 (4/4)	100 (4/4)	+ (2/2)			
Tg(HuAPP695.SWE)2123H	46	NA	100 (2/2)	100 (2/2				
Tg(HuAPP695.SWE)1844F	42	NA	100 (1/1)	100 (1/1)	+ (1/1)	-		

Table 5 (continued)

Clinical and pathological features of FVB mice expressing APP transgenes

Line	Copy Number (mean±SEM)	Transgenic brain APP (mean±SEM)	% sick or dead at 100 days	% sick or dead at 200 days	Cortico-limbic gliosis (in affected mice)	Extracellular HuAPP deposits	Weight (gm±SEM)	
							Tg	Non-Tg
Tg(HuAPP695.SWE)1837F	42	NA	100 (1/1)	100 (1/1)	+ (1/1)	-		
Tg(HuAPP695.SWE)1827F	59	NA	100 (1/1)	100 (1/1)	+ (1/1)	-		
Tg(HuAPP695.SWE)1665F	244	NA	100 (1/1)	100 (1/1)	+ (1/1)	-		
Tg(HuAPP695.DUT)2012	47±3.4	NA	100 (3/3)	100 (3/3)	(/2)			
Tg(HuAPP695.WTmyc)6214	4±0.3		0 (n=25)	0 (n=25)				
Tg(HuAPP695.WTmyc)466	40±9.0	1.0±0.21	0 (n=12)	33 (1/3)	- (0/1)			
Tg(HuAPP695.WTmyc)6209	28±6.1	1.6±0.43	35 (5/15)	75 (9/12)				
Tg(MoAPP695.WT)1859	6±0.8	0.8±0.21	0 (n=)	0 (n=2)				

Table 5 (continued)

Clinical and pathological features of FVB mice expressing APP transgenes

Line	Copy Number (mean±SEM)	Transgenic brain APP (mean±SEM)	% sick or dead at 100 days	% sick or dead at 200 days	Cortico-limbic gliosis (in affected mice)	Extracellular HuAPP deposits	Weight (gm±SEM)	
							Tg	Non-Tg
Tg(MoAPP695.WT)1869	26		75 (3/4)	100 (4/4)	+(1/2)			
Tg(MoAPP695.WT)1874	31±3.7	3.1±0.55	47 (8/17)	79 (11/14)	+(3/5)			
Tg(MoAPP695.WT)1855	29	2.7±0.1	0 (n=2)	100 (2/2)				

Behavioral analyses

To determine whether FVB mice naturally became behaviorally impaired with advancing age (the mouse equivalent of senile dementia in humans), FVB mice were observed up to one year and the behavior of these aged mice compared to that of transgenic mice. Behavioral analyses were usually performed three times per week using the corner index (CI) test. The test exploits a striking neophobic response which occurs in many affected Tg mice. The neophobic response is manifested by a decrease in exploratory activity specific to testing in a novel chamber. Early in the clinical course, affected mice often appear normal in their home cages but exhibit transient immobility for 30 to 60 seconds after being placed alone in a clean cage, in contrast to unaffected mice which typically explore and sniff around the novel setting. A characteristic response of an affected mouse is to hold its neck low with its tail stiff during the transient immobility. Alternatively, an affected mouse runs to a corner and then assumes a crouched or frozen posture there. The (CI) test measures the number of times a mouse sniffs the corners of a clean cage during the first 30 seconds after it is placed alone into that cage. Based upon the collective observations of >2000 tests of >100 Tg mice and >2500 tests of >140 non-Tg mice, we established criteria for the presence of a behavioral disorder were determined to be scores of two "0's" or "0 and 1" occurring within three consecutive tests. The onset of illness is ascribed to the first of three consecutive testing dates in which abnormal scores are obtained.

To perform the corner index test, a test mouse, held by the tail, is placed in the center of a clean cage that is otherwise identical to its home cage. The number of times the mouse sniffs the corners of the test cage during the first 30 seconds after it was placed into that cage are recorded as the CI. Animals which are obviously moribund before attaining the CI criteria and animals which develop witnessed seizures also are diagnosed as ill. Animals housed alone are excluded from the analysis because several non-Tg and Tg mice obtain low scores while housed alone without displaying the characteristic freezing postures of the affected Tg animals. When these mice are housed with other mice, their CI scores

increase. To control the variations in diurnal activity, all animals are tested between 1430h and 1830h.

An age-related CNS disorder in FVB mice

Behavioral abnormalities.

The life expectancy of FVB mice is approximately 600 days but little is known about age-related CNS disorders in FVB mice. To determine whether FVB mice naturally become behaviorally impaired with advancing age, 110 FVB mice 150-500 days of age from three different institutions (University of Minnesota, Minneapolis, MN, McLaughlin Research Institute, Great Falls, MT, and Harlan Sprague Dawley, Inc. Indianapolis, IN) were observed. With advancing age, 18 mice as early as 154 days of age developed behavioral abnormalities, including agitation, inactivity, seizures, and neophobia, as defined by the corner index test, and premature death (Table 6). Another six mice died from tumors or accidentally. Although agitation or inactivity occurred in all affected Tg mice, these were subjective signs that rarely appeared in most normal mice. the onset of illness was defined by corner index test results in conjunction with the observation of seizures, agitation or apathy. Both male and female mice were affected. Three agitated mice died prior to diagnosis by corner index criteria. One death occurred immediately following an observed seizure. The remaining mice grew progressively less active, and were sacrificed for pathologic studies between nine and 91 days after the onset of abnormal behavioral signs. The cumulative incidence of behavioral abnormalities and death (excluding accidental and tumor-related deaths) in this cohort of FVB mice was 23% by 500 days of age (See Figure 9).

Gliosis. Brains from sixteen older non-Tg FVB mice nine to twelve months of age, seven exhibiting the abnormal behavior characteristic of affected Tg APP mice and nine age-matched behaviorally normal mice, were examined in a coded fashion. Six of the seven brains from the behaviorally abnormal mice exhibited profound hypertrophic astrocytic gliosis in the hippocampus, prprhippocampal area, amygdala, and cerebral cortex (Figure 10). None of the brains from the nine age-matched, behaviorally normal mice exhibited this degree

of gliosis, although moderate gliosis restricted to the hippocampus was observed in some mice. These findings indicate that the behavioral disorder in affected older non-Tg mice is tightly associated with cortico-limbic gliosis (Yates-corrected $X^2=8.96$, $p=0.003$). The brains of the non-Tg behaviorally impaired FVB mice showed no amyloid plaque deposition, neurofibrillary tangle formation, neuronal abnormalities, or qualitative changes in neuronal or glial numbers.

Regional-cerebral glucose utilization. To obtain an independent functional assessment of the abnormal behavior observed in impaired FVB mice, regional brain glucose utilization was determined using a modification of the Sokoloff method (Sokoloff et al., (1977) *J. Neurochem* 28, 897-916). Regions associated with learning, memory, and emotion such as the cerebral cortex, hippocampus, entorhinal cortex, and amygdala, which are most impaired in cognitively impaired aged humans and patients with AD were examined. Densitometric values of ^{14}C-deoxyglucose distribution were normalized to cerebellar values because the cerebellum appeared uninvolved clinically and pathologically. The regional cerebral glucose utilization in cerebral tissue in impaired FVB mice was compared to that in cerebral tissue in behaviorally normal, age-matched FVB mice. Significant decreases ($p<0.05$, analysis of variance) in regional glucose utilization, particularly in the hippocampus (-42%), amygdala (-43%), entorhinal cortex (-46%), parietal cortex (-34%), frontal cortex (-19%) and temporal cortex (-18%), were observed in the cerebral tissue in the impaired FVB mice. In contrast, no significant decreases were observed in several structures, including the corpus callosum, medullary reticular formation, dentate nucleus, and vermis.

The development of impaired behavior accompanied by cortico-limbic hypertrophic gliosis and diminished regional cerebral glucose utilization, especially in the cerebrum, in FVB mice defines a characteristic age-related CNS disorder with features of the senescent changes observed in other rodent species, such as hypertrophic gliosis and diminished regional glucose utilization in limbic and cortical structures. Although the age-related behavioral abnormalities observed in impaired FVB mice have not been described to occur naturally in other rodents, the major decrease in regional cerebral glucose utilization found in the cortico-

limbic areas of the brain involved in learning, memory, and emotion, strongly suggest that some, if not most, of the behavioral abnormalities in affected FVB mice reflect dysfunction in these brain regions. Because the behavioral, pathological, and functional abnormalities observed in these mice share features found in other aged, impaired rodents and in demented humans, the constellation of findings represents a form of CNS senescence in FVB mice.

Transgenic mice expressing mutant and wild-type APP

Behavioral abnormalities. An abnormal phenotype resembling that in aged, impaired FVB mice developed in animals expressing high levels of APP. Copy number *per se* was unlikely to be the direct cause of the CNS disorder, since a previously published Tg line developed in FVB mice, Tg(HuPrP)FVB-152, expressing human PrP driven by 30-50 copies of the hamster PrP gene cosmid exhibited no premature behavioral abnormalities or death (Telling et al., (1994) *Proc.Natl.Acad.Sci. U.S.A.* 91,9936-9940). The phenotype in TgAPP mice segregated according to the species, genotype and level of APP expression in four lines harboring roughly equivalent copy numbers (20-30): Tg(HuAPP695.WYmyc)466, Tg(MoAPP695.WTmyc)6209. to determine whether PrP levels were affected by the presence of supernumerary PrP gene components, brain PrP levels were measured in Tg(HuAPP695.TRImyc) 1130 mice with 74 transgene copies and non-Tg mice. No differences were found, indicating that alterations in PrP expression were also not the cause of the abnormal phenotype.

Affected Tg animals developed all the clinical signs observed in aged, impaired non-Tg FVB mice, including agitation, increased startle responses, apathy, and neophobia (Table 6), but they occurred with significantly high penetrance at earlier ages (Figure 9, Table 5). Later in the course inactivity and failure to reproduce developed but there was no tremor, incoordination, weakness, paralysis, or apparent loss of sensation as judged from their withdrawal or vocal responses to tail or foot pinching. Seizures were observed in a small percentage (3% (6/181)) of affected Tg(HuAPP695.TRImyc) mice. It is possible that the actual incidence of seizures is higher, and would be detected if mice were observed for more than 30-60 seconds three times per week.

Behavioral abnormalities in Tg mice developed as early as one month of age. There was no significant difference between the onset of behavioral abnormalities in male and female mice. Some Tg mice (=14%) overexpressing APP died as early as one month of age without exhibiting prior seizures or neophobia. A neuropathologic examination of two of these mice identified cortico-limbic gliosis indistinguishable from Tg mice that had died after exhibiting the characteristic behavioral signs, so it is probable that these mice died as a result of the same disorder as the other affected Tg mice.

Small stature was observed in animals with transgenic brain APP levels exceeding twice the endogenous levels (Table 5). This difference in size was not apparent at birth became conspicuous by four to six weeks of age, and was less or absent in older animals. The Tg animals appeared normally proportioned. Small size was no required for behavioral abnormalities to occur, since Tg(HuAPP695.TRImyc)1118 mice died prematurely and developed behavioral abnormalities despite being normal in size.

Table 6.

Clinical and pathological signs in aged, impaired FVB mice and in affected FVB mice expressing APP transgenes

Signs	% aged, impaired FVB mice	% affected Tg FVB mice
Seizures	17% (3/18)	3% (6/181)
Agitation or inactivity	100% (18/18)	100% (181/181)
Neophobia	83% (15/18)	84% (152/181)
Early death (excluding sacrificed mice)	100% (4/4)	100% (82/82)
Cortico-limbic gliosis	86% (6/7)	76% (16/21)

Pathological analyses of transgenic mice

Brains of transgenic mice exhibiting behavioral abnormalities or found dead and age-matched nontransgenic littermates were examined for neuropathologic abnormalities. Brains were immersion fixed or perfused with 10% phosphate-buffered formalin or 4% buffered paraformaldehyde, embedded in paraffin, and cut into 5-8 μm sections on a rotary microtome. Tissue sections were stained with hematoxylin and eosin, cresyl violet, thioflavin S, or Congo Red stains, or using the Bielschowsky silver or TUNEL (Gavrieli et al., (1992) *Journal of Cell Biology* 119, 493-501) methods.

For immunohistologic studies, paraffin sections were deparaffinized and rehydrated through xylol and graded alcohols. Endogenous peroxidase was quenched by treatment with 6% hydrogen peroxide in methanol for 10 minutes or with 3.0% hydrogen peroxide in methanol (1:5), and rinsed in deionized water or phosphate buffered saline. To enhance APP antigen detection, selected sections were microwave irradiated in water at full power for 15 minutes, cooled to room temperature, transferred to deionized water in 0.5 M TBS (pH 7.6), and pretreated with 0.4% TX/TBS, followed by 3% normal goat serum in TBS. Primary antibodies 6E10 (1:100) and 8E5 (1:100 ascites fluid) were prepared in 0.1% TX/TBS with 2% normal goat serum.

Following incubation for 24 hours, slides were rinsed, incubated in goat-antirabbit or -antimouse IgG (1:20) in 0.1% TX/TBS, and rinsed in TBS followed by one-hour incubation in rabbit or mouse peroxidase-antiperoxidase (1:100) at room temperature. Rinsed slides were reacted in the presence of 0.05% diaminobenzidine in 0.01% hydrogen peroxide, rinsed three times in TBS, dehydrated through a graded series of alcohols to xylene. Representative sections were silver-enhanced according to the Fontana-Masson method (Masson (1928) *Am. J. Path.* H:181-211), and viewed under transmitted light microscopy and differential interference contrast optics. Other sections were immersed in 70% formic acid for 10 minutes, rinsed in phosphate buffered saline, and immersed in 10% normal horse serum for 1 hour. Primary antibody 6E10 (1:5000) was prepared in phosphate buffered saline. Following incubation overnight at 4°C,

sections were rinsed in phosphate buffered saline, incubated with antimouse IgG, followed by avidin-biotin complex (Vector Labs, Inc). Rinsed slides were reacted with diaminobenzidine and counterstained with Harris hematoxylin. GFAP was detected using a monoclonal antibody to GFAP at a dilution of 1:60 in phosphate buffered saline.

Gliosis. Using coded specimens, brains from 21 affected Tg mice expressing the triple HuAPP variant, the Dutch HuAPP variant, the Swedish HuAPP variant, wild-type HuAPP, as well as brains from 12 age-matched, unaffected non-Tg mice were examined. Brains from 16 affected Tg mice exhibited prominent hypertrophic astocytic gliosis located predominantly in the parahippocampal area, hippocampus, amygdala, and cerebral cortex (Figure 10), with relative sparing of the basal ganglia. The astrocytes had enlarged, elongated processes when immunostained for glial fibrillary acid protein (GFAP), but there was no increase in the number of astrocytes. Brains from the age-matched non-Tg mice were devoid of the reactive gliosis, indicating a strong association between gliosis and abnormal behavior (Yates-corrected $X^2=14.83$, $p=0.00012$). Bielschowsky silver stains revealed no neurofibrillary tangles, dystrophic neurites, or neuritic plaques. Neurons appeared normal with Nissl and hematoxylin and eosin stains.

Gross and microscopic examinations of six Tg mice found dead revealed characteristic brain pathology (astrocytic gliosis in the hippocampus, cerebral cortex, amygdala, and parahippocampal area, as described below), but no evidence of microscopic or gross pathology outside the CNS. Amyloid was specifically excluded by thioflavin S staining in the heart, lung, liver, spleen, thymus, kidney, small intestine, and testes in four of these Tg mice. The absence of pathologic findings outside the CNS indicates that the deaths were most likely due to causes which were neurologic in origin.

Regional cerebral glucose utilization

To determine whether there were functional differences in the brains of affected Tg mice, regional brain glucose utilization was compared among affected Tg mice with aged, impaired non-Tg FVB mice and age-matched non-Tg mice.

Compared to normal, non-Tg littermates, significant reductions ($p<0.05$; analysis of variance) in glucose utilization were observed in various forebrain regions in Tg mice, including the hippocampus (-31%), amygdala (-28%), parietal cortex (-34%), temporal cortex (-33%), and occipital cortex (-36%). Many regions, in contrast, showed no significant reduction ($p>0.05$), including the sensory-motor cortex, corpus callosum, reticular formation, vermis, vestibular complex, and dentate nucleus. The diminution of regional glucose utilization was particularly pronounced in the hippocampus, amygdala, and some cortical regions in affected Tg mice closely resembling that occurring in older, impaired non-Tg FVB mice.

Extracellular Aß staining in a Tg mouse.

One animal shows extracellular staining with an antibody described in Saido et al., *JBC* 269 (21) :15253-15257, 1994. This antibody specifically stains the aminoterminus of Aß. It is an affinity purified polyclonal antibody. The staining in our Tg mouse can be blocked by specific competition with the Aß fragment. The staining pattern in our Tg mouse resembles that which is seen in AD brain stained with the same antibody. More animals are being examined. Further characterization with other antibodies is being done. Ultrastructural studies also being done.

Example 7

Testing for Drugs That Prevent Progressive Neurologic Disease

The animals of the invention are used to test materials for the ability to confer protection against the development of progressive neurologic disease. An animal exhibiting the progressive neurologic disease is treated with a test material in parallel with an untreated control transgenic animal exhibiting the neurologic disease. A comparatively lower incidence of the progressive neurologic disease in the treated animal is detected as an indication of protection. Treated and untreated animals are analyzed for diminished exploratory/locomotor behavior (CI test; see Example 6), as well as diminished 2-deoxyglucose uptake/utilization and hypertrophic gliosis in the cortico-limbic structures of the brain. To determine if a treatment can prevent or delay the onset of disease, half of the transgenic mice in

a litter from a line of mice known to develop neurologic illness may be randomly assigned to receive the treatment, and the other half to receive a placebo, beginning at an age prior to the earliest known onset of disease for the given line of mice. The number of litters to be used will depend upon the magnitude of the differences observed between treated and untreated mice.

Mice are observed daily; their diagnosis is facilitated by the use of the CI test (see Example 6) which is administered three times per week by individuals blinded to the experimental groups. Survival curves and mean ages of disease onset and death are calculated form the accumulated clinical data.

Clinical results are corroborated by performing neuropathologic and glucose-uptake studies in samples in the experimental and control groups. Gliosis is evaluated in immunohistologic studies using antibodies to glial fibrillary acidic protein. Glucose-uptake studies are performed using a modification of the Sokoloff method described by Chmielowska et al. (1986) *Exp. Brain Res.* 63:607.

To determine if a treatment can ameliorate or cure disease, sick littermates are randomly assigned to receive the treatment of interest or a saline placebo. Survival and clinical improvement on the CI test coupled with neuropathologic and glucose-uptake studies are ascertained, as described above.

Example 8

Testing for Drugs That Cure Progressive Neurologic Disease

The animals of the invention are used to test materials for the ability to improve or cure progressive neurologic disease. An animal exhibiting the progressive neurologic disease is treated with a test material in parallel with an untreated control transgenic animal exhibiting the neurologic disease. A comparatively delayed death, or an improvement in the neurobehavioral, pathologic, or functional indications of the disease is detected as an indication of protection. Treated and untreated animals are analyzed for diminished exploratory/locomotor behavior, as well as diminished 2-deoxyglucose

uptake/utilization and hypertrophic gliosis in the cortico-limbic structures of the brain.

As demonstrated by the above results, the clinical and pathologic findings in non-human mammals with super endogenous levels of either mutant or native amyloid precursor protein show an unexpected, but striking parallel to these in humans with progressive neurologic disorders such as Alzheimer's disease; the involved regions of the neocortex in affected transgenic mice and humans are similar. In addition, glucose uptake in the sensorimotor area of the cerebral cortex was unaffected by the neurologic disease in transgenic mice. This was the only region of mouse neocortex sampled which represented mainly primary neocortex, rather than a mixture of primary and association neocortex. It is a well-known observation that in brains of patients with Alzheimer's disease, the primary neocortex is relatively free of neuropathologic findings compared to the association cortex.

The CNS phenotype of the Tg mice closely resembles the CNS phenotype of a subset of aged non-Tg mice of the same FVB strain. The gliosis in the hippocampus astrocytic gliosis that is characteristically found in the hippocampal formations of aged, memory-deficient rats (Landfield et al. (1977) *J. Gerontology* 32, 2-12) and aged, nude mice (Mandybur et al., (1989) *Acta Neuropathol (Berl.)* 77, 507-513). The regional glucose hypometabolism in both the affected Tg mice and the aged, impaired non-Tg mice was markedly diminished in the hippocampus, cerebral cortex, and amygdala, resembling the pattern of glucose gypometabolism occurring in humans with AD (de Leon et al. (1983) *Am J. Neuroradiology* 4, 568-571), and in restricted areas of the limbic system in aged, impaired Sprague-Dawley rats (Gage et al. (1984) *J. Neuroscience* 11, 2856-2865). The striking similarities in the neurologic disease exhibited by the Tg animals and the naturally occurring disorder in older mice of the same strain support the use of these Tg mice as a model for progressive senescent disorders of the brain, including Alzheimer's disease.

Animals dying of neurologic disease exhibited hypertrophic gliosis in the hippocampus, amygdala, and some areas of the cerebral cortex. Immunohistologic

mapping of HuAPP in the transgenic mice indicated widespread expression throughout the brain. However, the behavioral abnormalities corresponded to the circumscribed regions of gliotic pathology and glucose hypo-utilization found in select forebrain regions. The striking similarities in target cell specificities in cortico-limbic areas of the brain (hippocampus, amygdala, and some areas of cerebral cortex) in these transgenic mice and Alzheimer's disease support the use of these transgenic mice as a model for progressive neurologic disorders such as Alzheimer's disease.

In summary, these transgenic mice express supra-endogenous levels of APP. In the lines which develop neurologic disease, APP transgene product expression with at least 200% of endogenous levels have been attained, or more than double that reported in any prior publications. More importantly, these mice have a definite, progressive neurologic disorder. Even where APP expression has been achieved in other transgenic mice, they have not developed a progressive disease affecting the cortico-limbic areas of the brain.

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

What is claimed is:

1. A method for making a transgenic non-human mammal with progressive neurologic disease in corticolimbic areas of the brain, said method comprising chromosomally incorporating an expression cassette which comprises an amyloid precursor protein coding sequence operably joined to regulatory sequences obtainable from a prion protein gene which provide for expression of said coding sequence in neurologic tissues at a level at least two to four-fold that of endogenous levels of wild-type amyloid precursor protein into the genome of a non-human mammal.

2. The method according the claim 1, wherein said coding sequence is a human coding sequence.

3. The method according to claim 1 or 2, wherein coding sequence is a disease-linked mutated coding sequence.

4. The method according to claim 3, wherein said coding sequence is selected from the group consisting of CS1HuAPP695.V717I.V721A.M722V.; CS2HuAPP695.V717I.V721A.M722V.; and CS1HuAPP695.K670N.M671L.

5. The method according to any one of claims 1-3 wherein said coding sequence is a chimeric coding sequence.

6. The method according to claim 5 wherein said chimeric coding sequence is a human-mouse chimeric coding sequence.

7. The method according to any one of claims 1 to 6, wherein said regulatory sequence includes a sequence from ACCATG, ACCATG, ACCATGG, and ACCATGG, wherein ATG is the initiation codon.

8. The method according to any one of claims 1 to 7 wherein said expression cassette comprises a region of DNA flanking the prion regulatory sequences whereby copy number-dependent transgene expression is obtained.

9. The method according to any one of claims 1 to 8 wherein said progressive neurologic disease is a neurobehavioral disorder with gliosis and one or both of diminished glucose uptake and glucose utilization.

10. The method according to any one of claims 1 to 9 wherein at least twenty copies of the expression cassette are incorporated into the genome.

11. A zygote of a non-human mammal comprising:

an expression cassette as defined in any one of claims 1 to 8.

12. A zygote according to claim 11 wherein said zygote is derived from crossing rodents of the same species.

13. The zygote according to claim 12, wherein said rodents are mice.

14. The zygote according to claim 13, wherein said mice are FVB mice.

15. The zygote according to claim 13, wherein said mice are different strains.

16. The zygote according to claim 15, wherein said strains are a Swiss Webster and a CS7B16/DBA-ZF1 hybrid.

17. An embryo developed fro the zygote according to any one of claims 11-16.

18. An animal developed from the embryo according to claim 17.

19. A transgenic non-human animal whose germ cells and somatic cells comprise an expression cassette which provides a level of expression of a mutant amyloid precursor protein gene which produces progressive neurologic disease in cortico limbic areas of the brain of said mammal, wherein said expression cassette is as defined in any one of claims 1 to 8.

20. The cosmid vector comprising an expression cassette as defined in any one of claims 1 to 8.

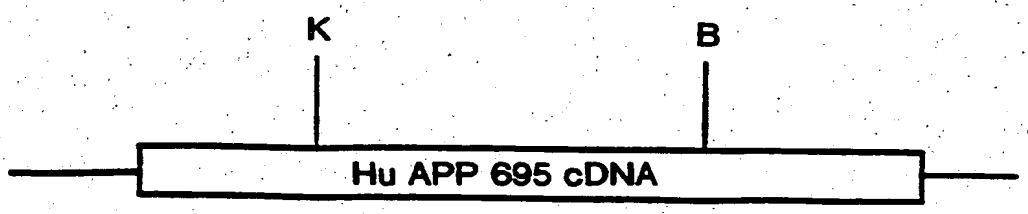
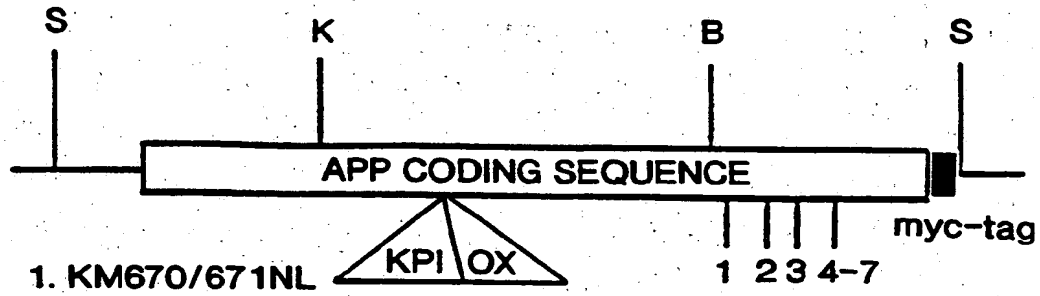
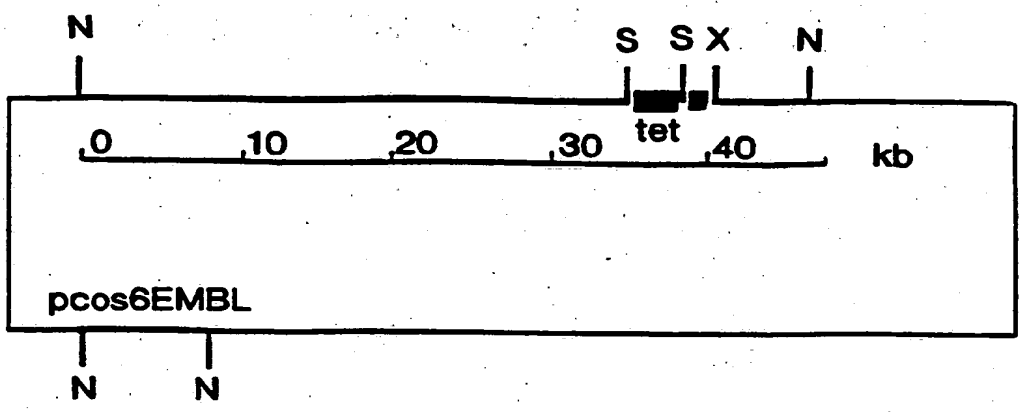


FIG. 1



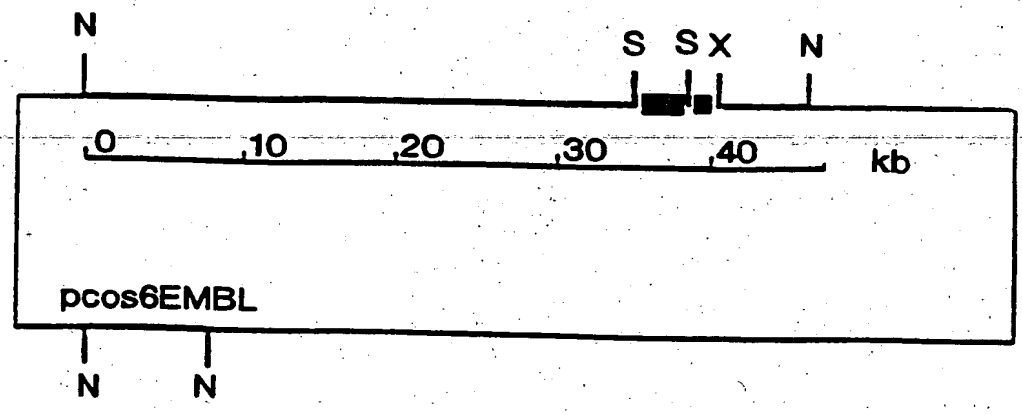
1. KM670/671NL
2. A692G
3. E693Q
4. V717I
5. V717G
6. V717F
7. VVM717/721/722IAV

FIG. 2



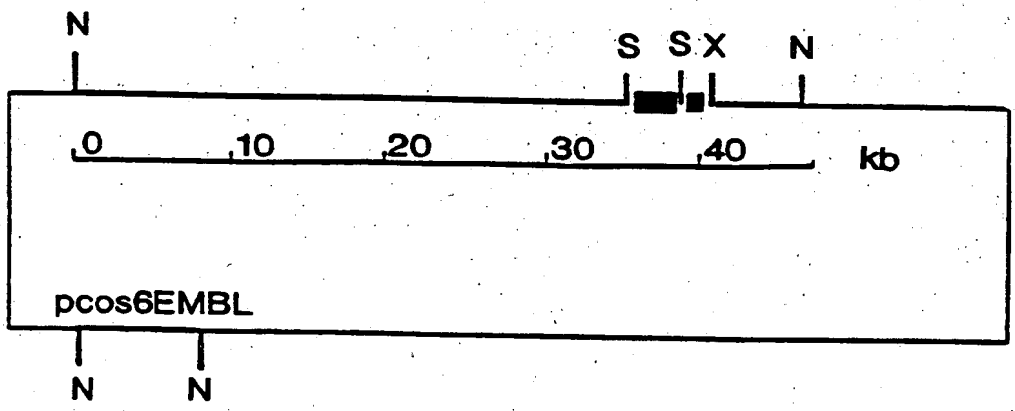
▬ 1.6 kb 3'-UNTRANSLATED SEQUENCE

FIG. 3



APP CODING SEQUENCE WITH CS1 TRANSLATION INITIATION AS ILLUSTRATED IN FIG. 6

FIG. 4



APP CODING SEQUENCE WITH CS2 TRANSLATION INITIATION AS ILLUSTRATED IN FIG. 7

FIG. 5

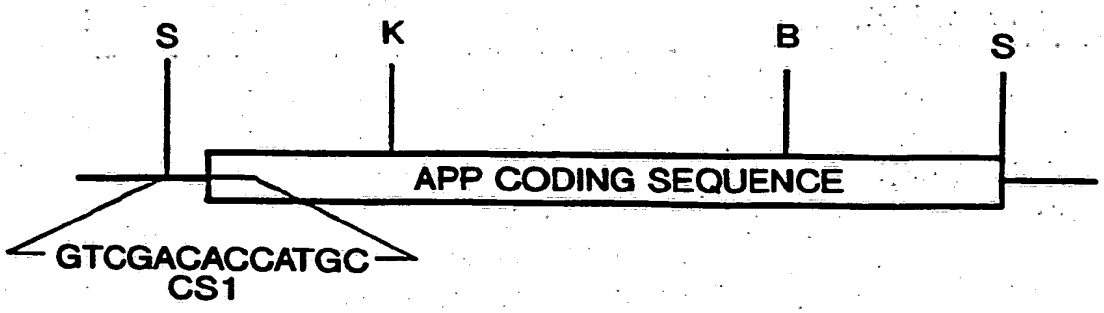


FIG. 6

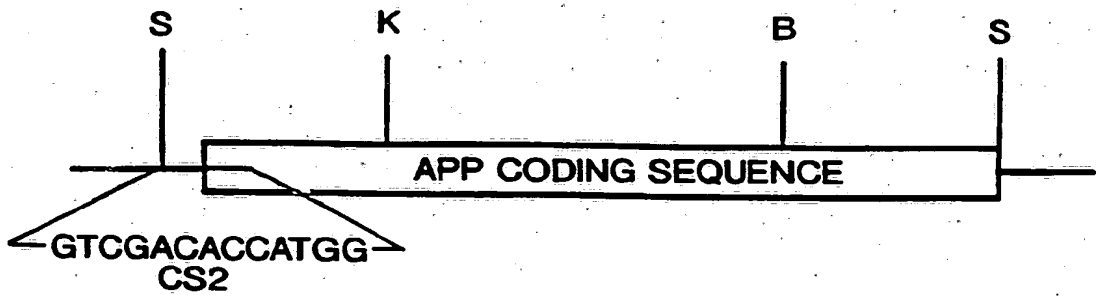
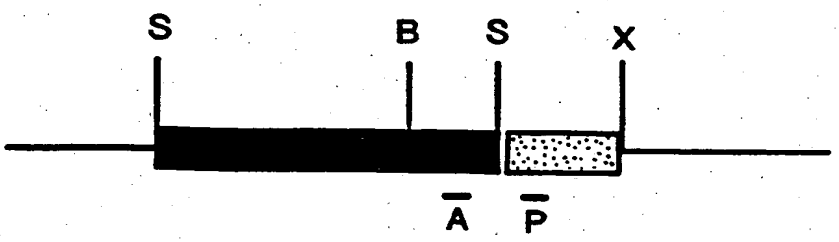


FIG. 7



A = CTG ACC ACT CGA CCA GGT TCT GGG T
P = GTG GAT AAC CCC TCC CCC AGC CTA GAC CA

APP CODING SEQUENCE

1.6 kb SEQUENCE FROM HAMSTER PrP 3'-UNTRANSLATED REGION

DNA SEQUENCES FROM THE HAMSTER PrP COSMID VECTOP AS ILLUSTRATED IN FIGS. 4&5

FIG. 8

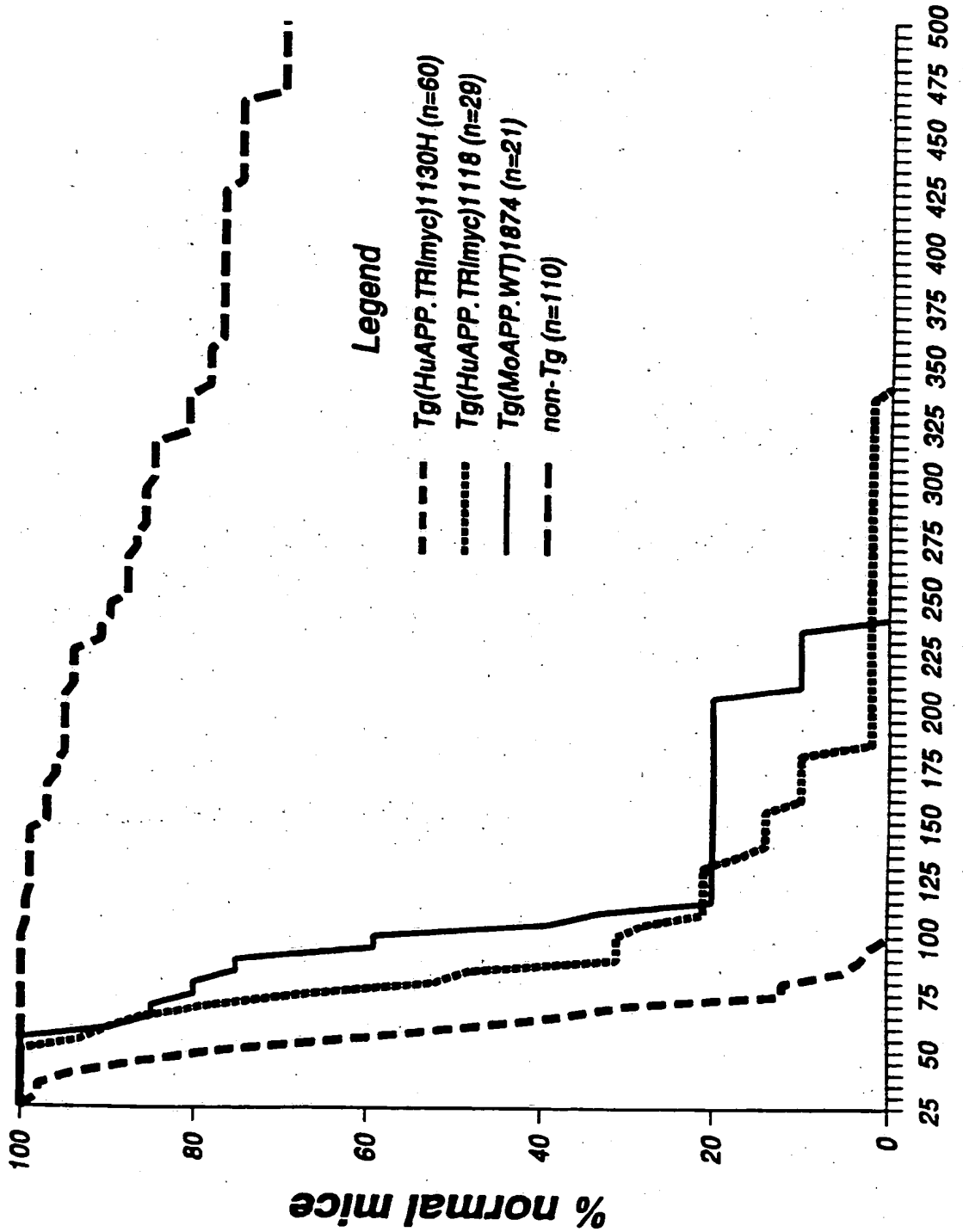


FIG. 9

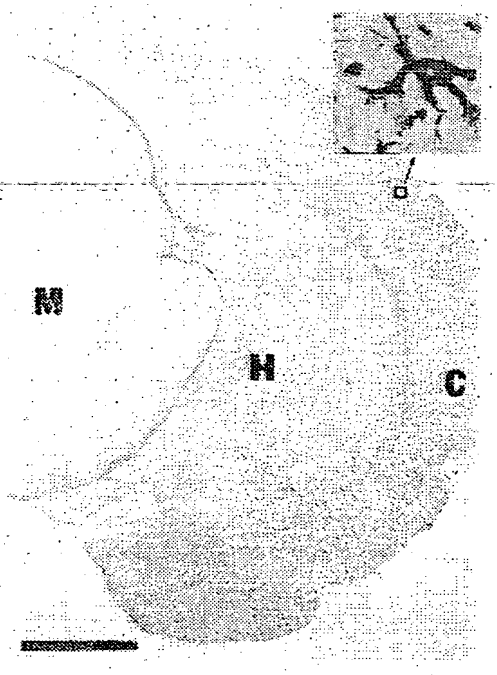


FIG. 10A

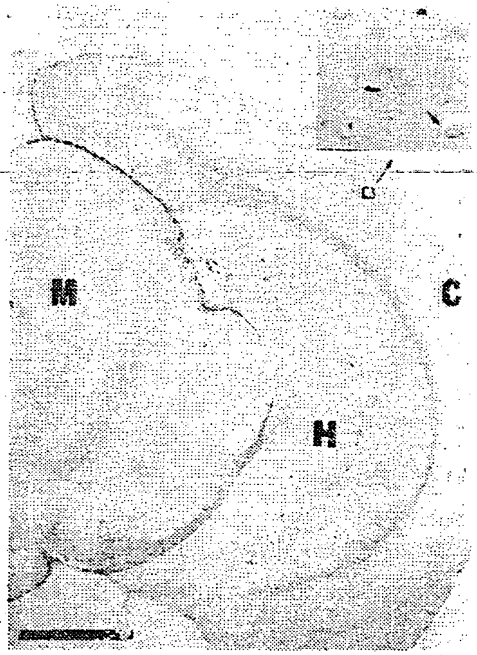


FIG. 10B

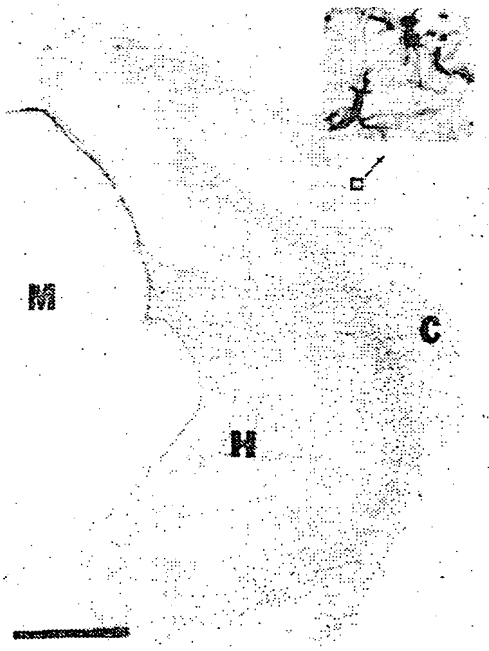


FIG. 10C

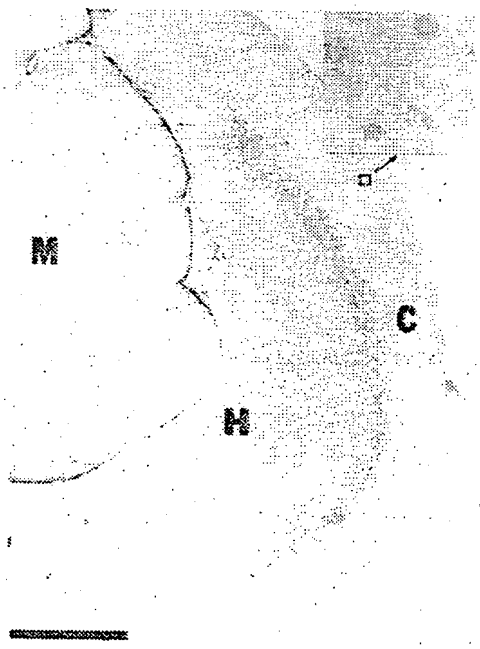


FIG. 10D

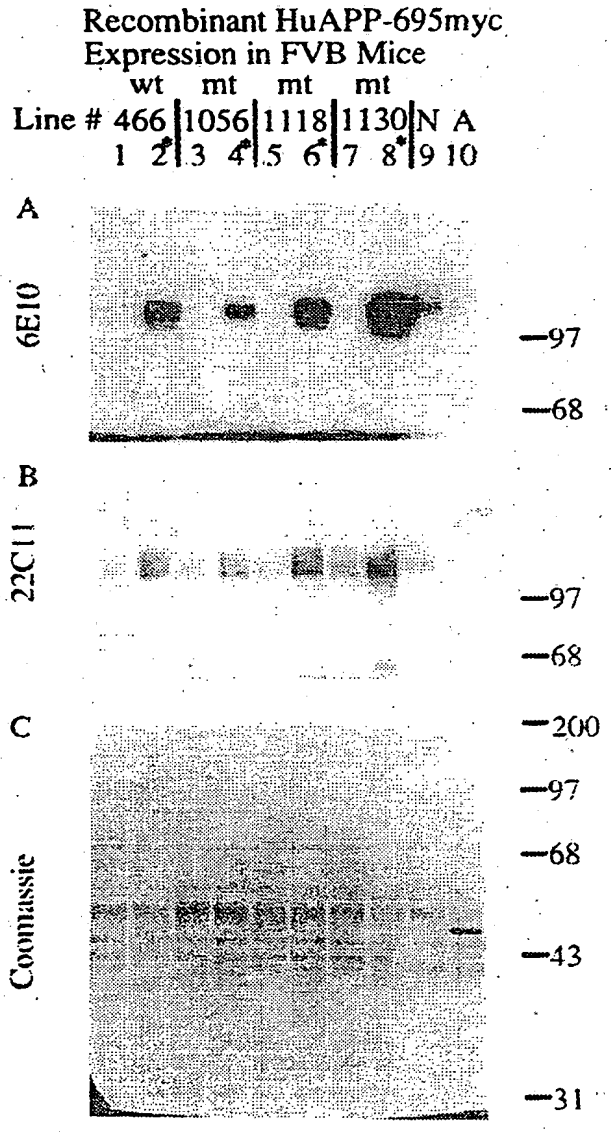


FIG. IIA

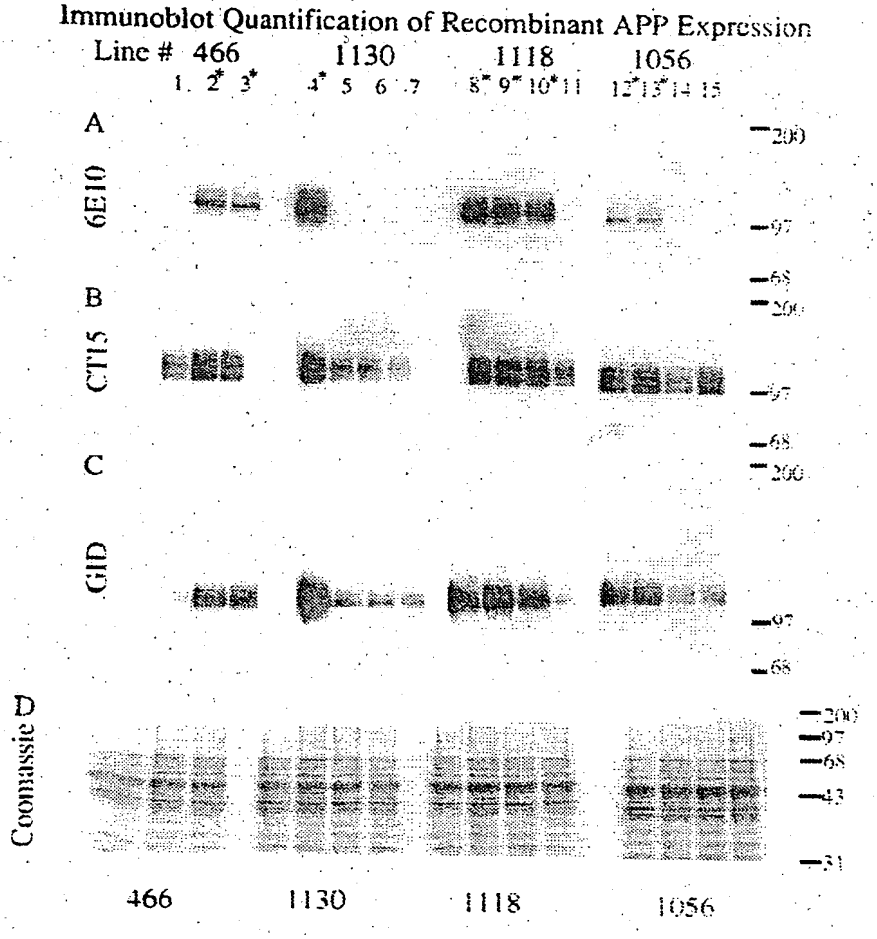


FIG. 11B

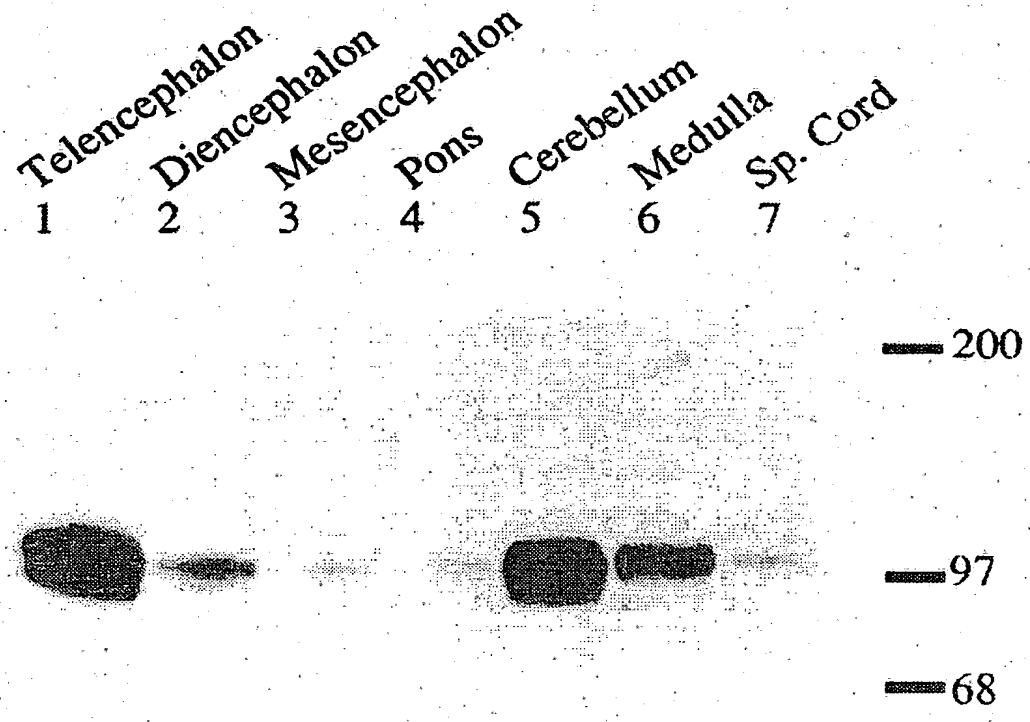


FIG. IIC

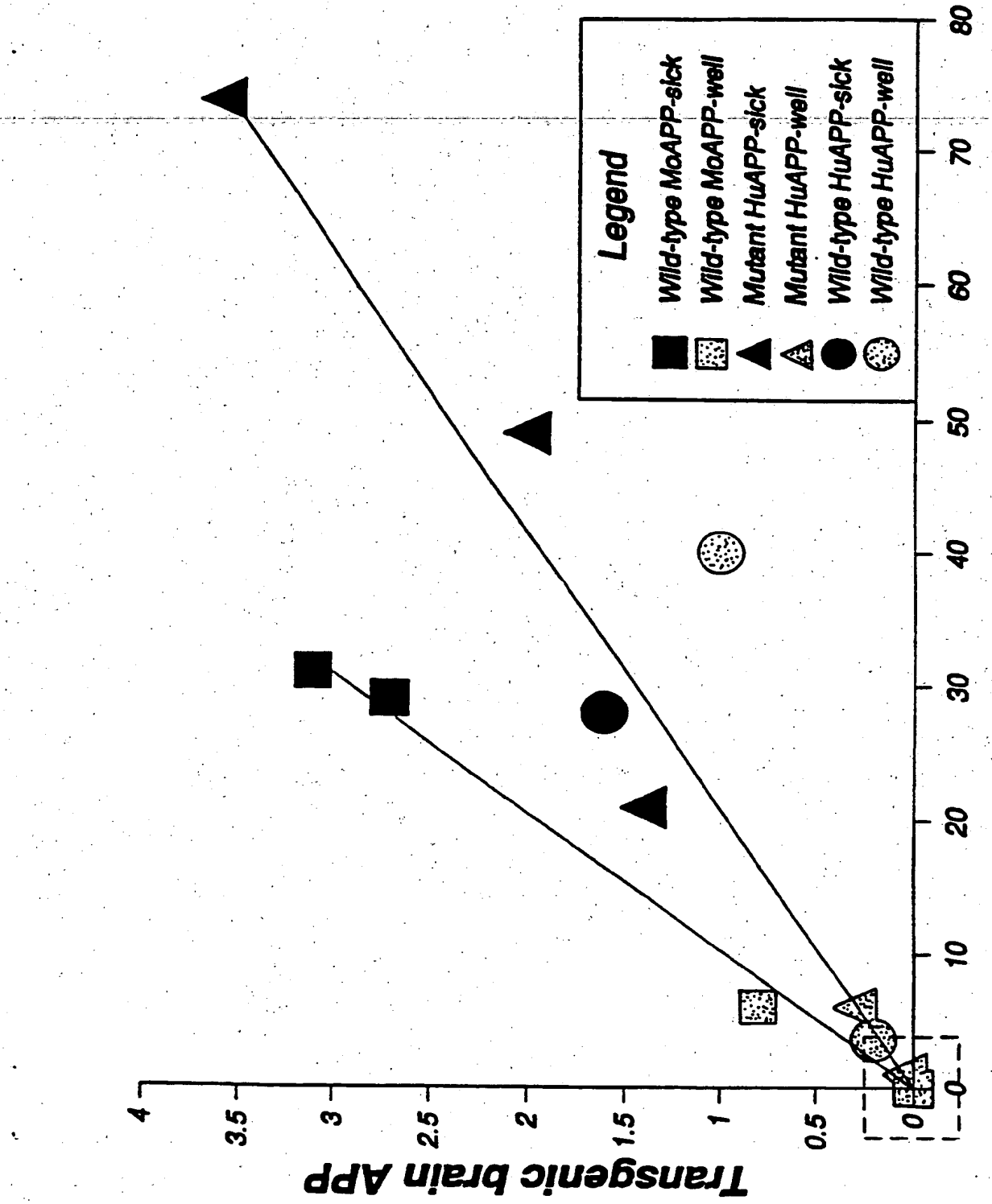


FIG. 12

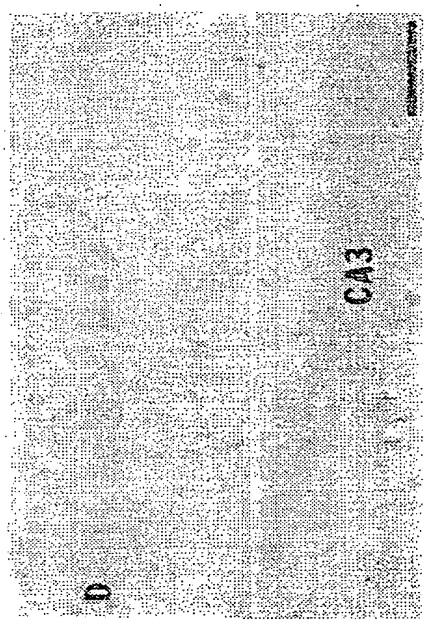


FIG. 13b

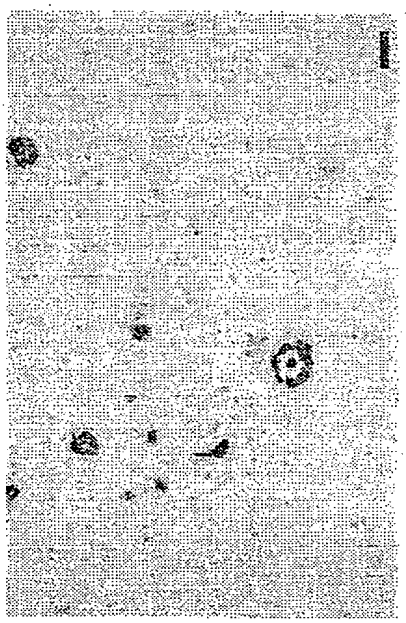


FIG. 13d

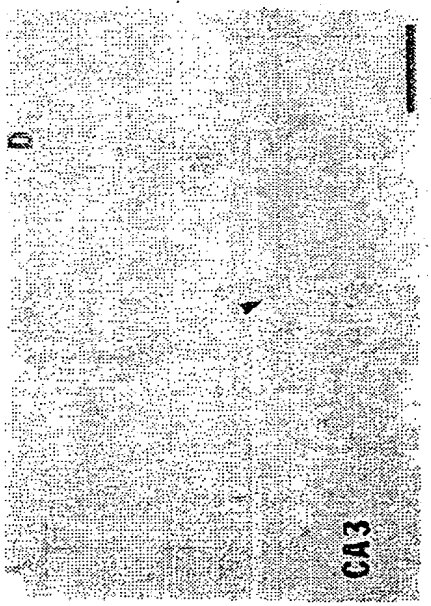


FIG. 13a

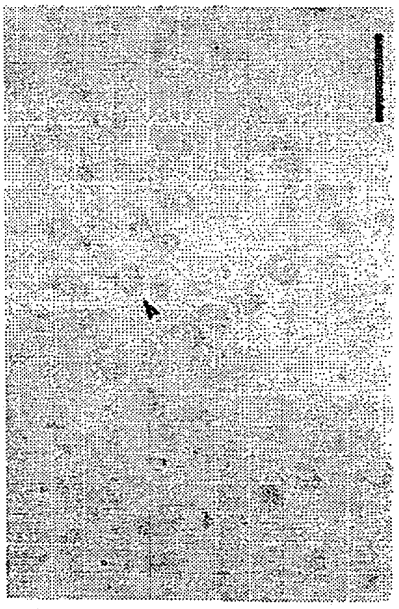


FIG. 13c

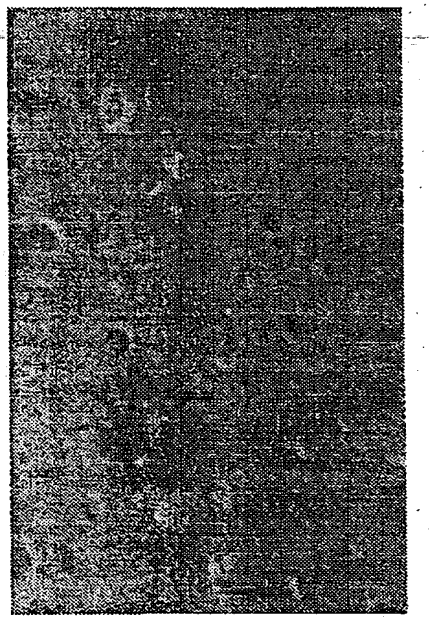


FIG. 13f

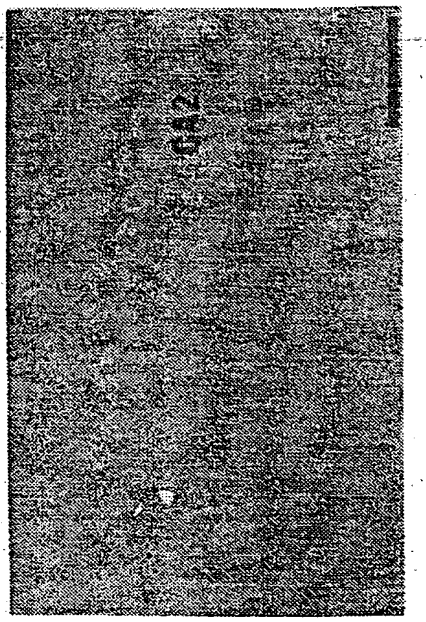


FIG. 13h

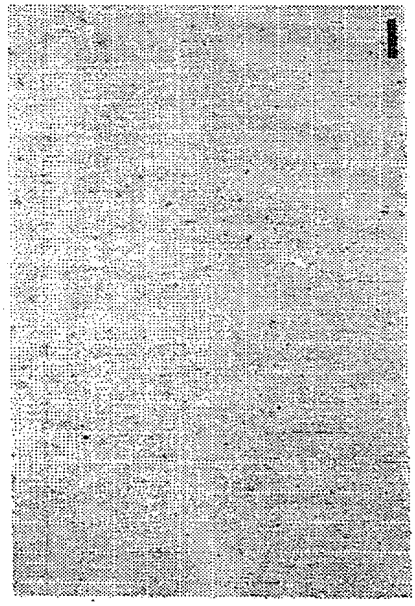


FIG. 13e

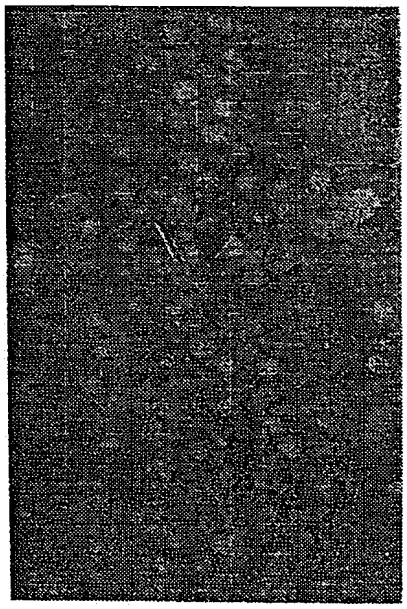


FIG. 13g

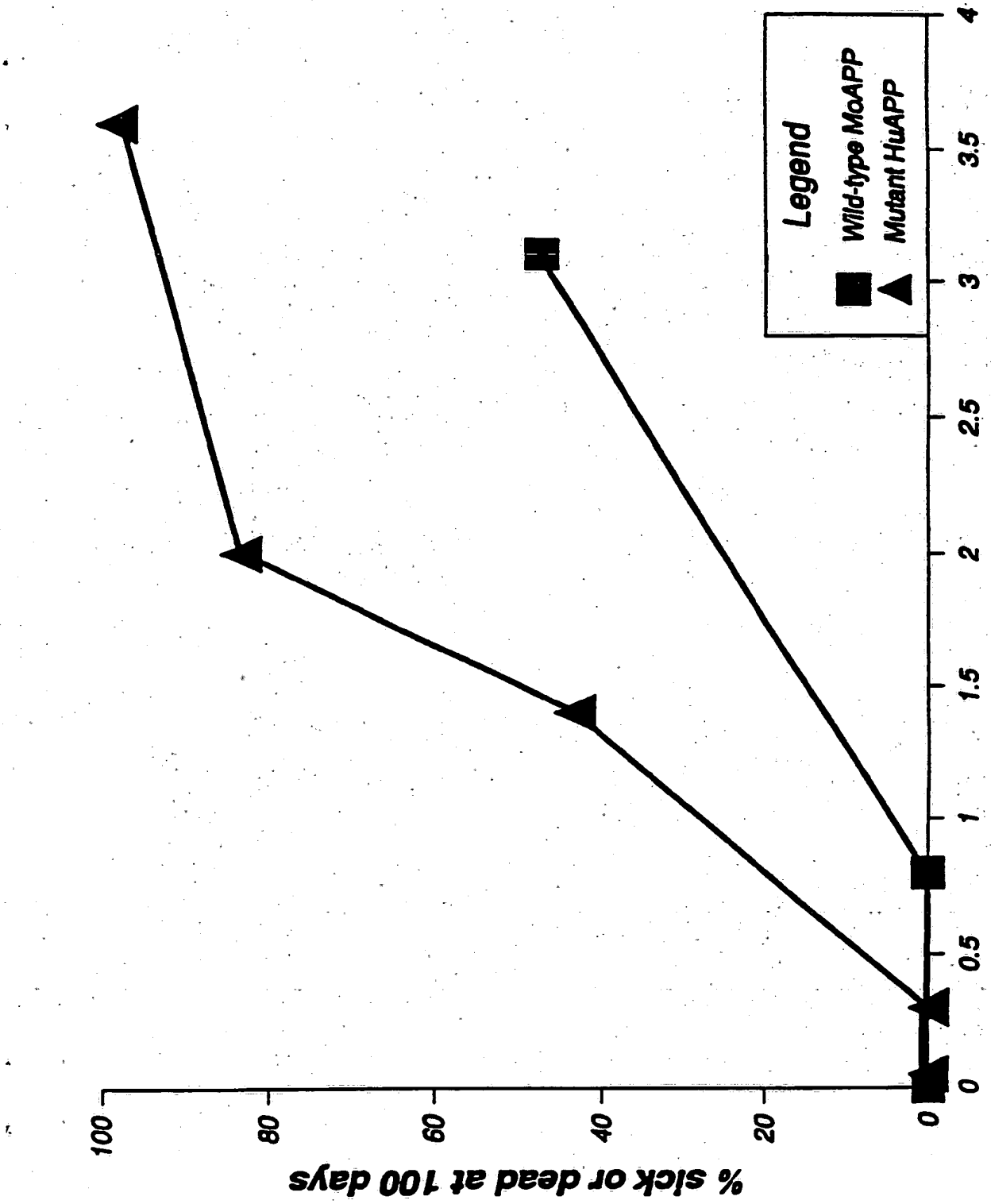


FIG. 14

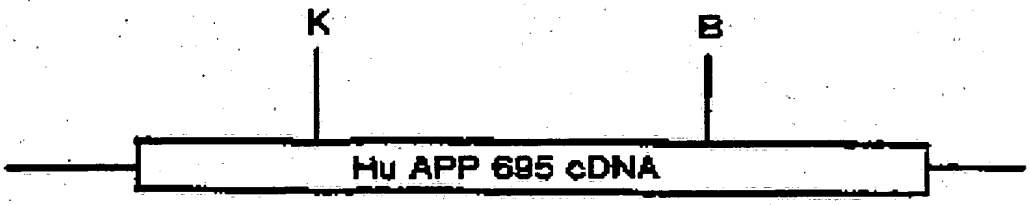
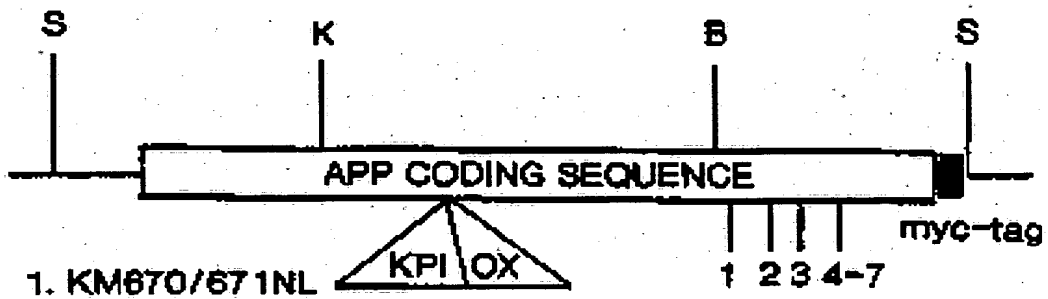
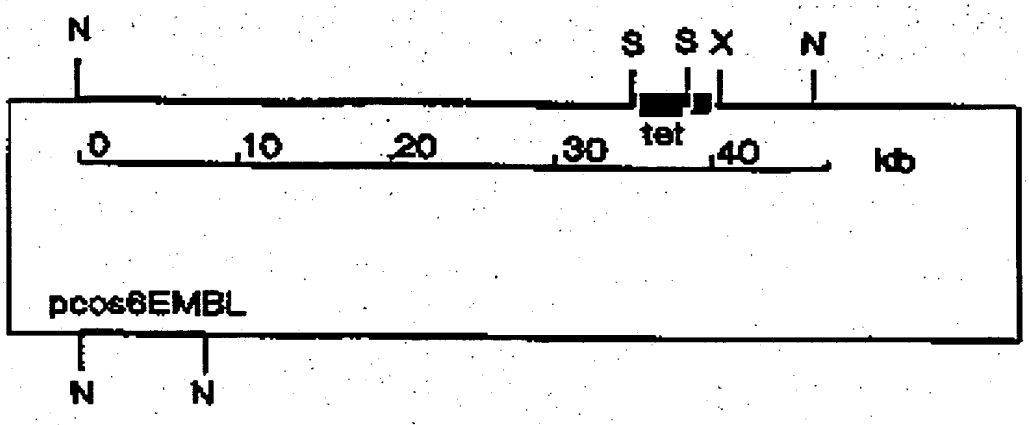


FIG. 1



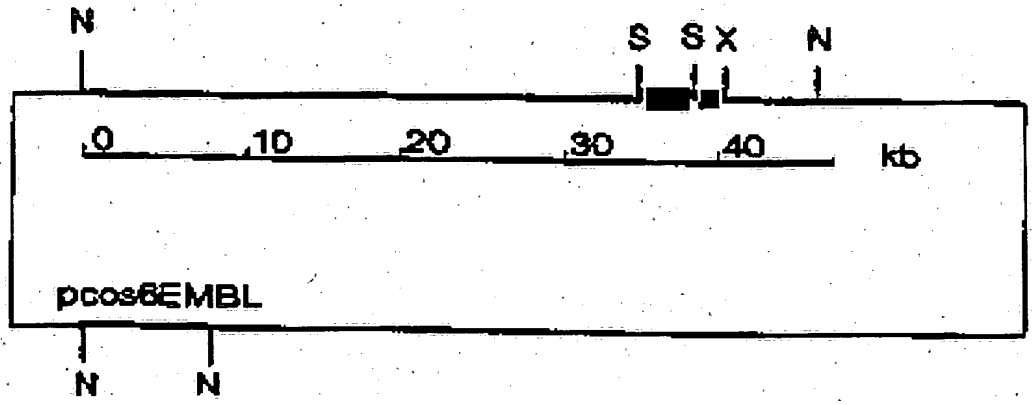
1. KM670/671NL
2. A692G
3. E693Q
4. V717I
5. V717G
6. V717F
7. VVM717/721/722IAV

FIG. 2



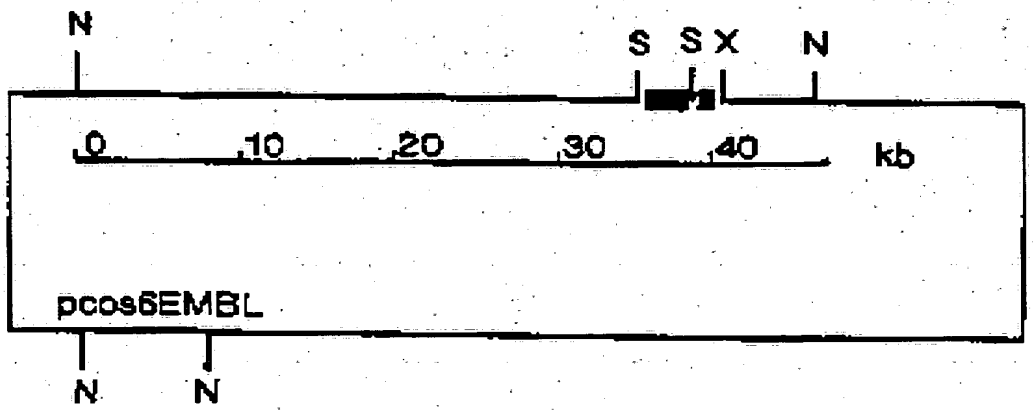
▬ 1.6 kb 3'-UNTRANSLATED SEQUENCE

FIG. 3



APP CODING SEQUENCE WITH CS1 TRANSLATION INITIATION AS ILLUSTRATED IN FIG. 6

FIG. 4



APP CODING SEQUENCE WITH CS2 TRANSLATION INITIATION AS ILLUSTRATED IN FIG. 7

FIG. 5

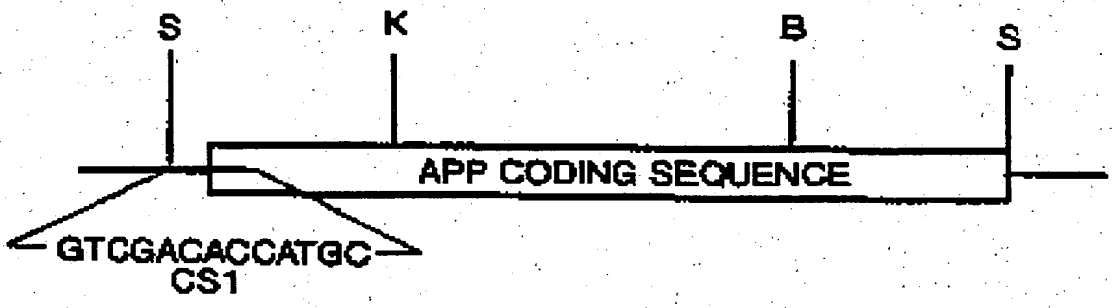


FIG. 6

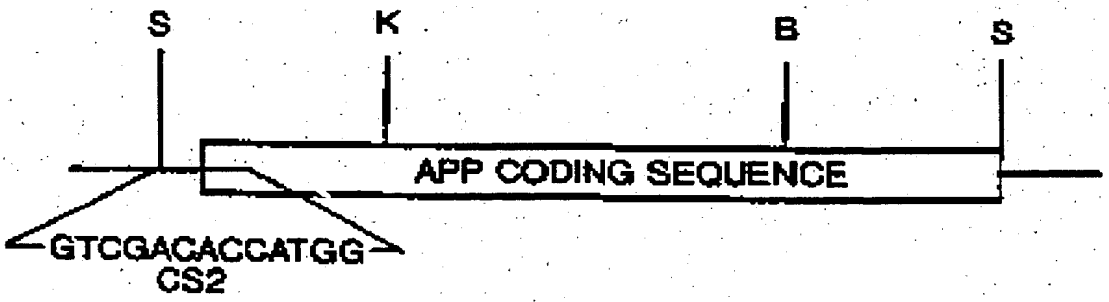
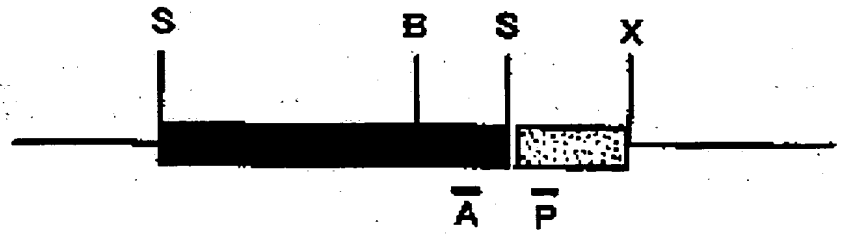


FIG. 7



A = CTG ACC ACT CGA CCA GGT TCT GGG T
P = GTG GAT AAC CCC TCC CCC AGC CTA GAC CA

APP CODING SEQUENCE

1.6 kb SEQUENCE FROM HAMSTER PrP 3'-UNTRANSLATED REGION

DNA SEQUENCES FROM THE HAMSTER PrP COSMID VECTOR AS ILLUSTRATED IN FIGS. 4&5

FIG. 8

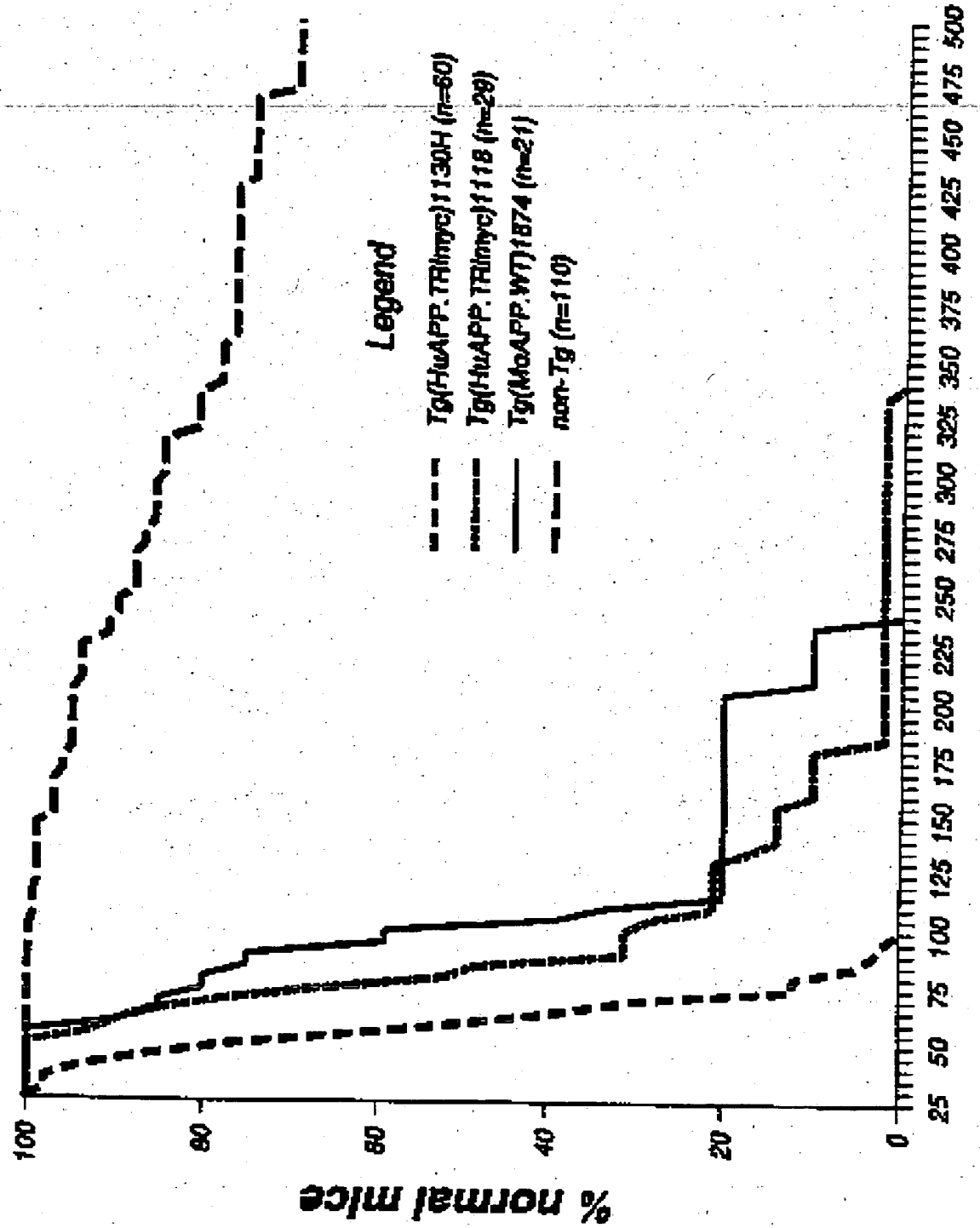


FIG. 9

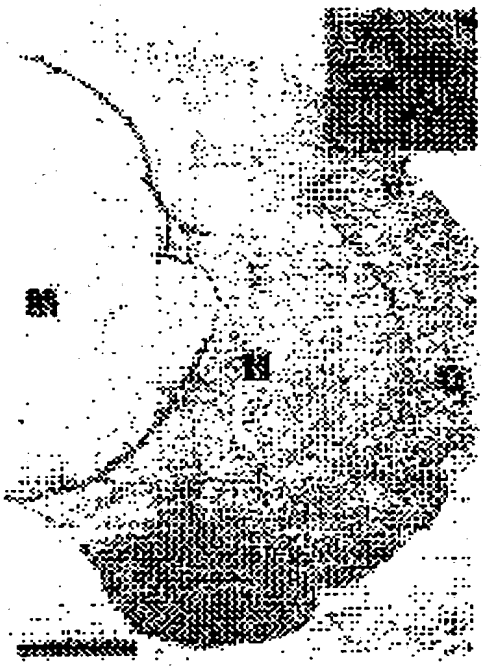


FIG. 10A

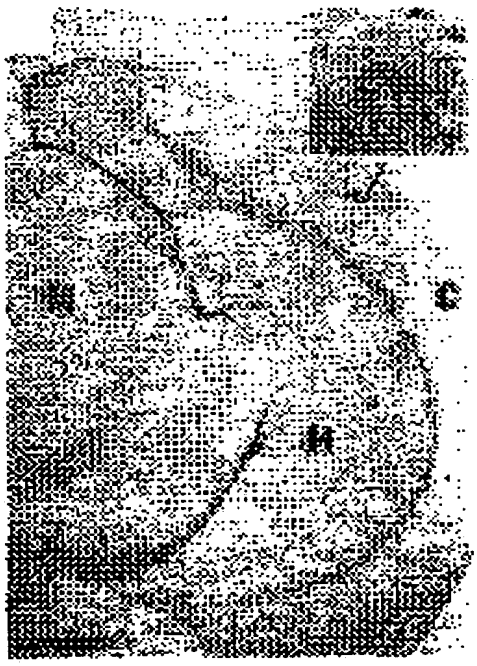


FIG. 10B

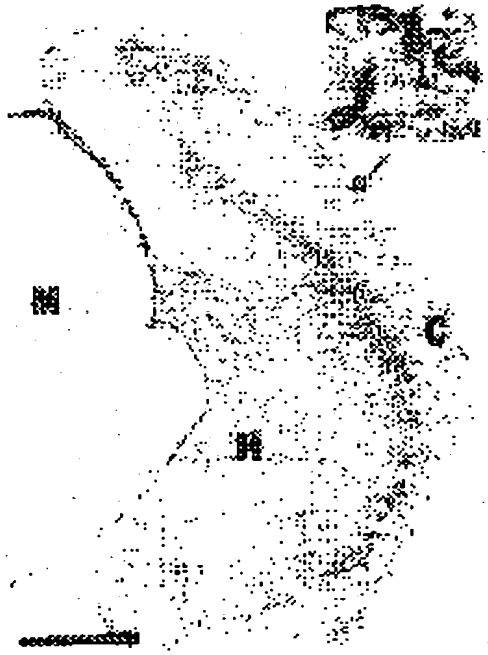


FIG. 10C

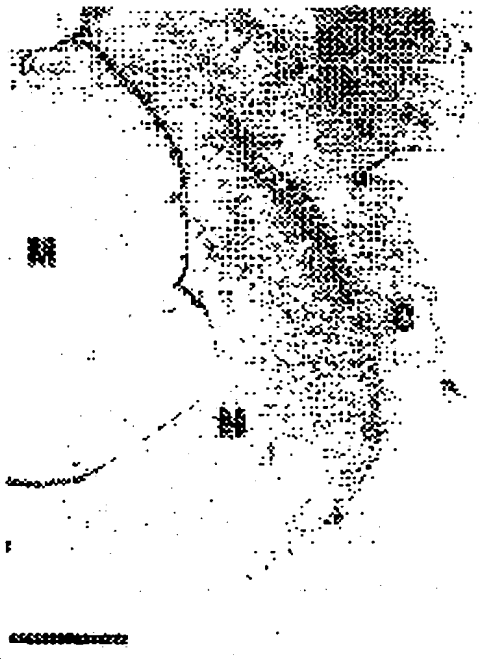


FIG. 10D

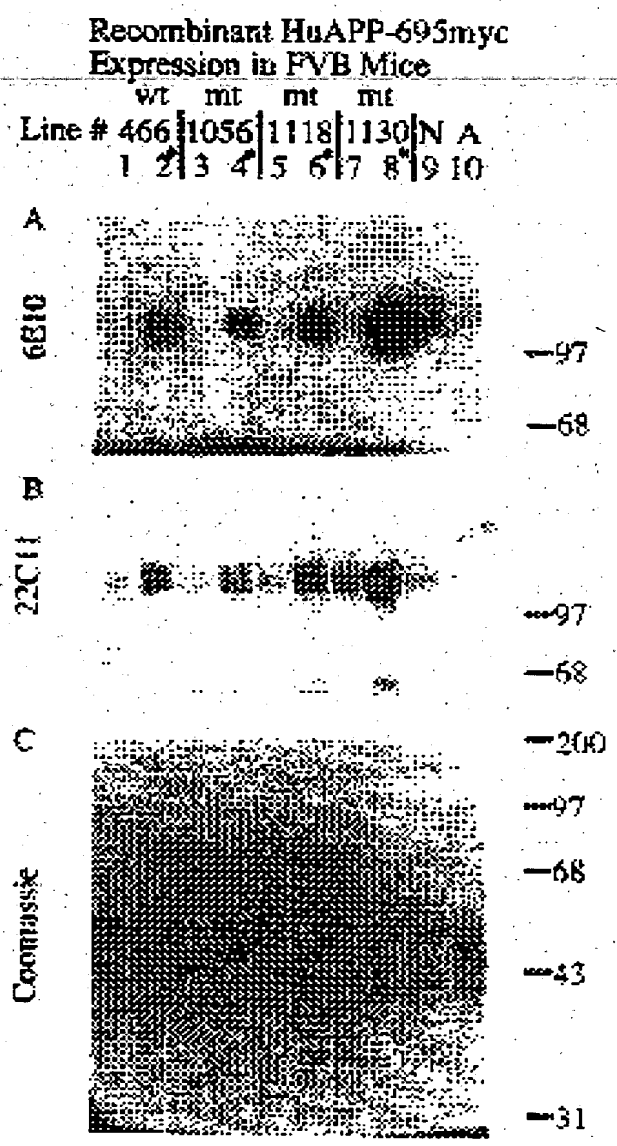


FIG. 11A

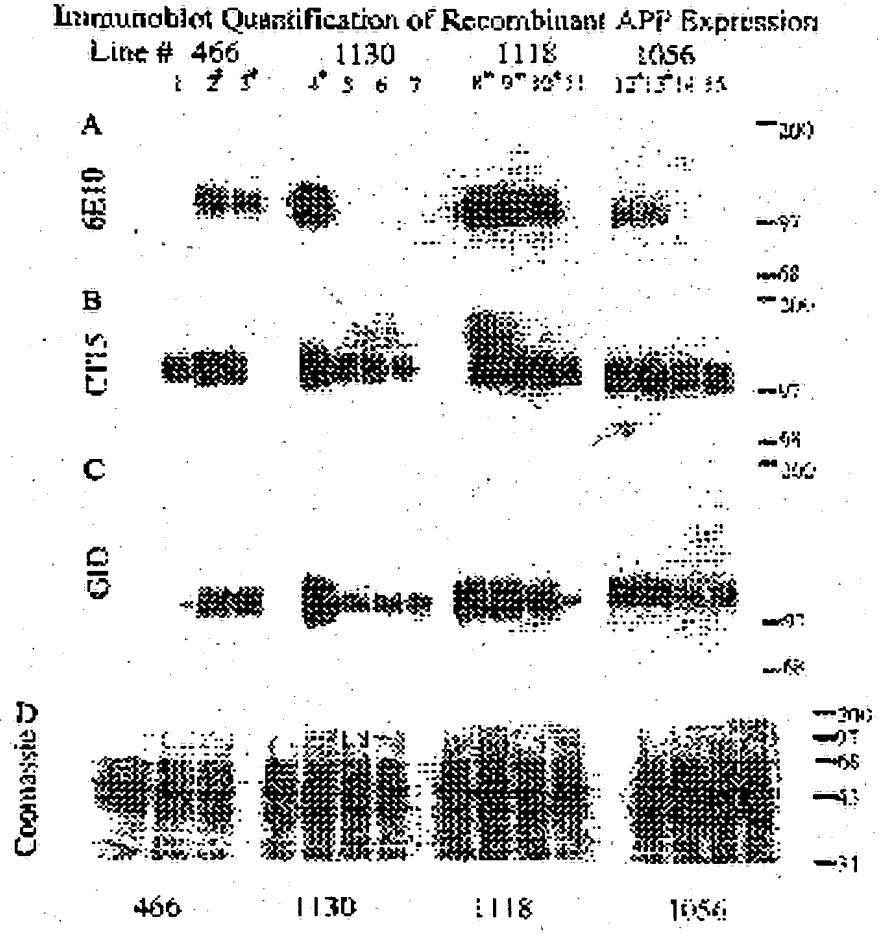


FIG. 11B

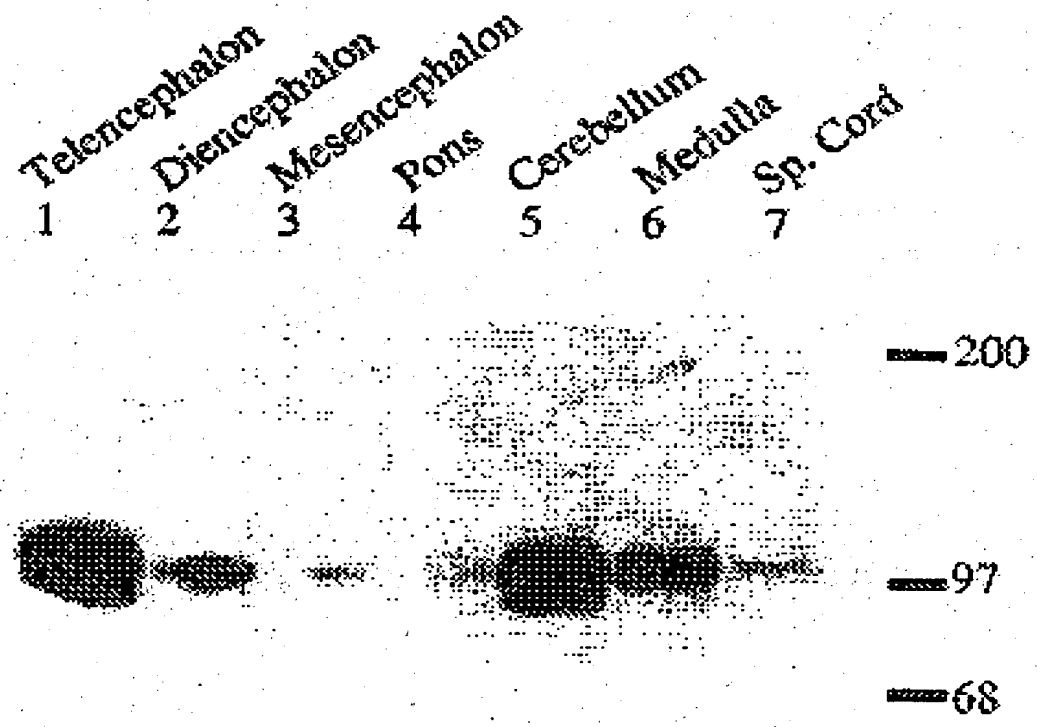


FIG. IIC

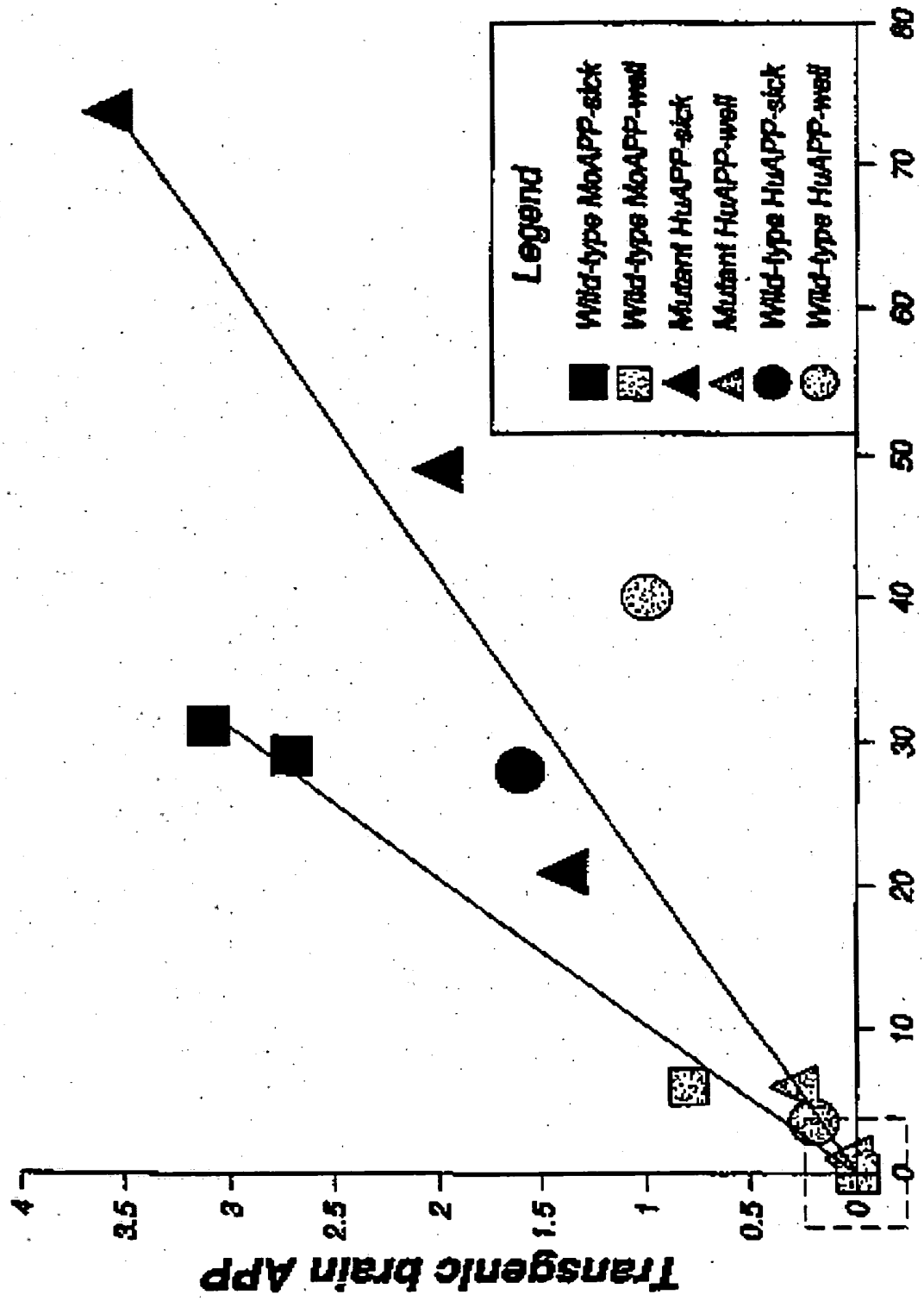


FIG. 12

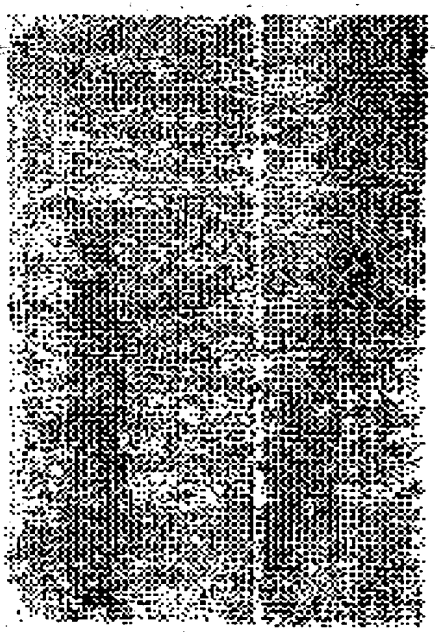


FIG. 13b

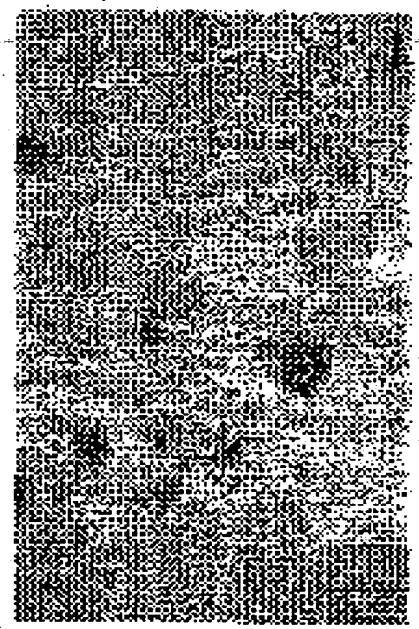


FIG. 13d

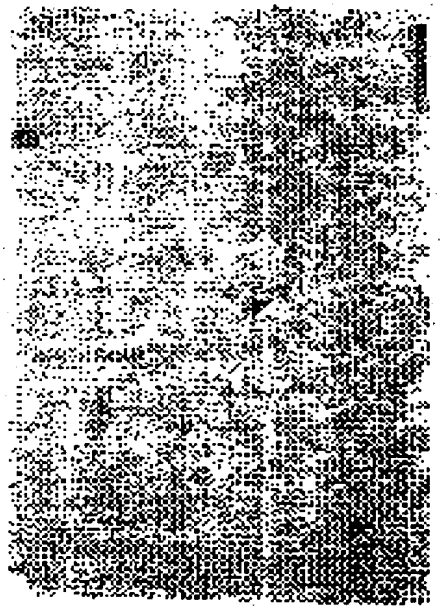


FIG. 13a

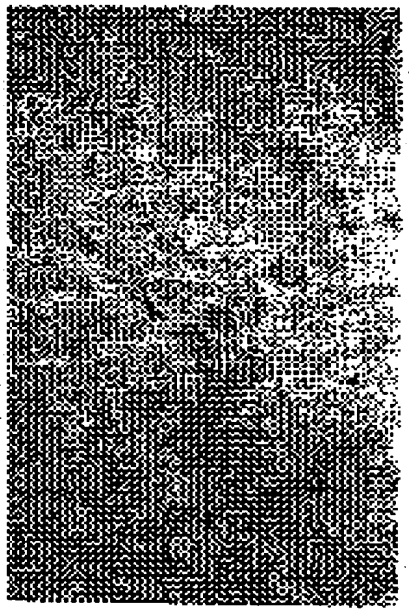


FIG. 13c

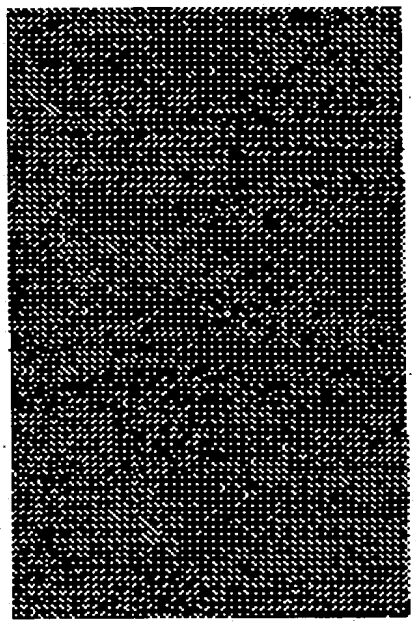


FIG. 13f

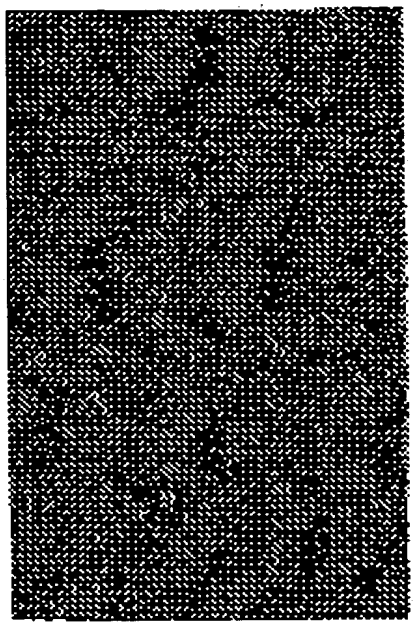


FIG. 13h

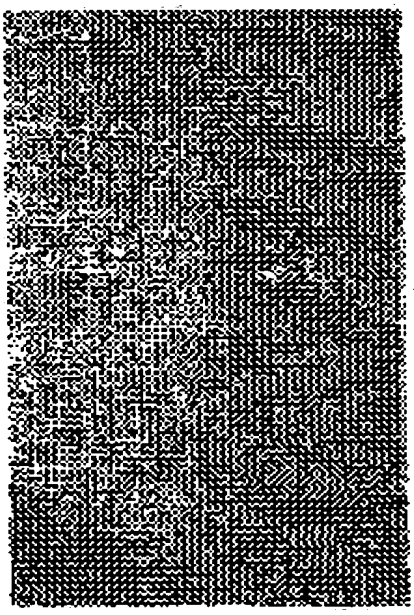


FIG. 13e

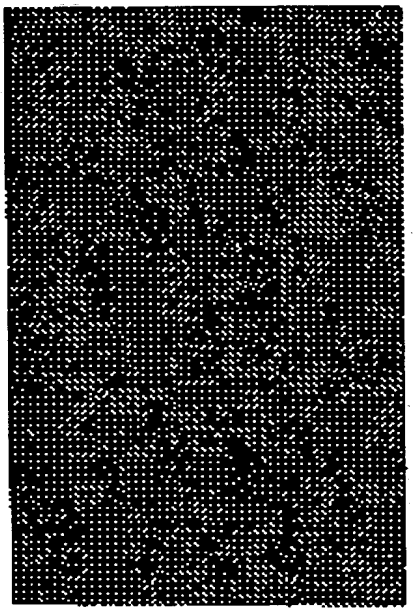


FIG. 13g

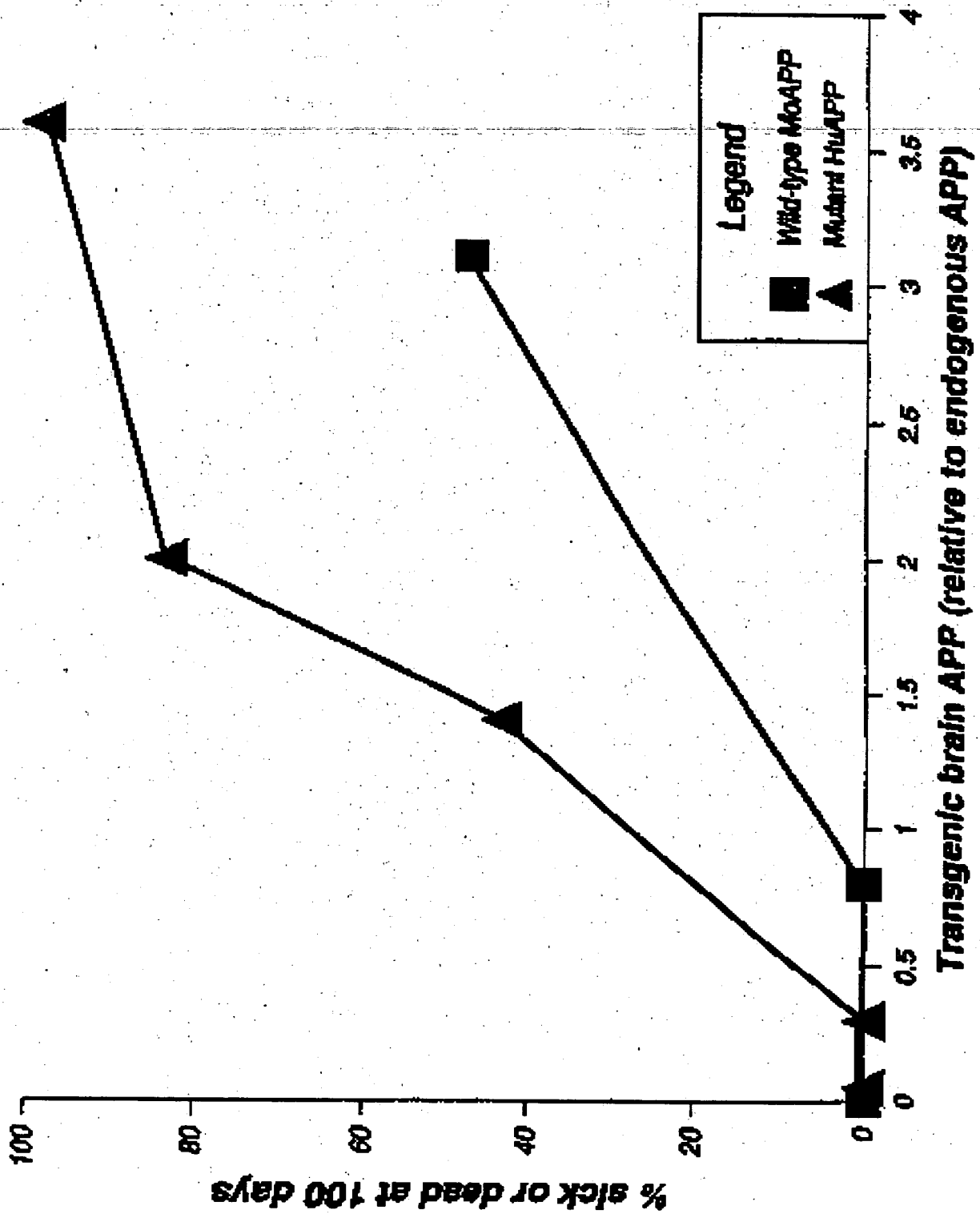


FIG. 14